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INTRODUCTION

The objective of these studies up to the current period has been to determine whether or not the degradation products following tissue injury, specifically thermal, can be lethal and toxic to the host; to isolate and identify these "toxins;" to determine whether these "toxins" have the capacity of producing "autoantibodies" in the injured host; and to determine if the blood of patients convalescing from extensive thermal injury will counteract the toxemia and reduce the mortality of severely burned human subjects.

The objective of the current period has been to produce toxic and lethal substances from burned normal human skin in vitro.

The objective for the future is to isolate and identify the "toxins" produced in vitro; to produce immunization artificially against these "toxins" in animals for the treatment of acutely burned subjects; and to determine whether these "toxins" can be used for active immunization.

1. STUDIES UP TO THE CURRENT PERIOD THAT LED TO THE USE OF BURNED CONVALESCENT BLOOD IN ACUTELY BURNED HUMAN SUBJECTS, INCLUDING RECOMMENDATIONS FOR TREATMENT.

Since 1928, when the senior investigator was responsible for a burn service in a children's hospital, he was impressed by the high degree of toxicity associated with extensive burns, which toxicity indicated to him that a "toxin" related to the burning of the skin and other tissues might be involved and should be investigated.

In 1934, he showed experimentally in shoats and guinea pigs as well as in human subjects that following burning of the skin, a "toxin" was present in the serum.(1) At the same time, however, he also showed that after healing, in animals and man, there appeared substances in the serum which would neutralize this "toxic" effect.(2) This was published in 1937, and according to Feoderov (3), this was the first time that neutralizing substances following thermal injury were described and published.

In subsequent studies in the senior investigator's laboratory, it was found that in tissue injury, whether it be physical or chemical, at least two components must be considered. One is concerned with the primary tissue damage related directly to the injury, and the second is due to the sequeilae of the primary injury. In vitro studies in this laboratory have demonstrated that the degradation products resulting directly from thermal insult to the skin cannot be inhibited by proteolytic enzyme inhibitors (such as soy bean trypsin inhibitor, sodium salicylate, etc.). On the other hand, the secondary degradation products can be influenced by proteolytic enzyme inhibitors. (4) In in vivo studies in mice which were subjected to thermal injury, a similar phenomenon prevailed; i.e., following the injury, the mortality rate was high in the first 24 to 48 hours, but after that the mortality rate decreased; nevertheless, death did occur over a period of 20 to 30 days, depending upon the severity of the burn. The basket technique was used for scalding, thus eliminating gangrene and infection.(5)

These studies imply that whereas degradation products ("toxin") are poured into the blood stream immediately after injury in large quantities, similar substances continue to pour into the blood stream as long as the wound remains open. The primary injury has liberated or

activated numerous enzyme systems which in turn cause degradation of the tissue to continue. This basic concept is paramount in the understanding of the pathogenesis of the toxic and lethal effects of injury and in the treatment of the latter with convalescent serum.

The complexity of the blood is legend. The non-specific toxin-neutralizing properties of the blood, its adsorbent characteristics, etc., necessitated a new approach to the problem of the isolation of a "toxin." A solution was found when it was learned that it is possible to produce a pocket under the rat skin that will remain intact for several days. By immersing the dome of this pocket that contained air into scalding water, it was reasoned that the material resulting from the injury to the skin would diffuse into such a pocket when fluid was contained therein.(6) By washing such pockets after thermal injury with saline or water, it was found that a series of pharmacologically active substances could be isolated, such as histamine, bradykinin, adenlyic compounds and possible serotonin,(7) and in addition a "toxin" that was toxic and lethal to rats and mice. This "toxin" was heat stable, dialysable and partially precipitated by 80% alcohol.(8)

This new method of collecting toxic substances from injured skin circumvented the circulation and allowed for the collection of large amounts of material needed for study and isolation. The material from these pockets, plus Freund's adjuvent, injected into rabbits produced sera of high titre.(9) The antibodies thus produced would prevent to a great extent the effects of the "toxin" in mice. It was also shown that in rats previously burned, autoantibodies were produced which reacted with the serum of acutely burned rats as well as to crude pocket "toxin."(10) Thus, the artificially produced antibodies in rabbits are related to the naturally occurring antibodies in rats and the artifi-

cially produced antigen or "toxin" in rats (pockets) is related to the "toxin" found in the acutely burned rats.

At this point, the tragic fire at Our Lady of the Angels School in Chicago took place. There, the senior investigator was given the opportunity to use convalescent serum of burned subjects in the most extensively and critically burned children. At the same time, studies were undertaken to determine if the blood of these acutely burned individuals contained toxic substances. Using HeLa cell cultures,[11] it was found that when a suspension of HeLa cells was mixed with acute burn serum before seeding (0.1 to 0.2 ml), inhibition of growth of these cultures took place. Normal serum failed to neutralize this effect. Mixing this acute sera with convalescent sera for one hour and then adding this mixture of suspensions to HeLa cells revealed that the toxic effect was partially or completely inhibited, depending upon the potency of the convalescent serum. In addition, blood drawn before and after transfusion with convalescent blood or plasma from these acutely burned patients, revealed that whereas the serum before the transfusion would inhibit the HeLa cell growth, the serum afterward would not do so.

Table I depicts the results of acute burn sera before and after transfusion of convalescent serum in four patients. It will be noted that in every case there had been inhibition before convalescent blood transfusion, and no inhibition after these transfusions.

A total of sixteen sera were tested from the school fire victims who had sustained from 19 to 61% body burns, and four who had sustained only multiple fractures without burning (ages 9 to 14). All but one demonstrated the inhibitory effect on HeLa cells.

TABLE 1

TISSUE CULTURE CELL GROWTH EXPERIMENTS USING SERIAL BLOOD SPECIMEN TAKEN BEFORE AND AFTER TRANSFUSION WITH BLOOD FROM HEALED BURNED DONORS

	PATIENT														
	1					2			3			4			
BLEEDING DAY (post burn)	1	14	15	19	31	2	16	21	2	11	16	10	16	22	
CELL GROWTH	-	-	+	+	+	-	-	+	-	-	+	-	+	+	
TRANSFUSION DAY		14	15	16			16	19			16		16		

The sera of fifteen naval recruits who were febrile and of seventy-two who were asymptomatic (Table 2) failed to inhibit the growth of HeLa cells, albeit many of the sera were allowed to remain on the clot for periods of up to two weeks at 4° C. This was done to simulate some of the conditions that prevailed with sera from the burned subjects.

In forty-nine individuals selected at random, the serum of only one demonstrated the neutralizing effects of acute burn sera on HeLa cells. A more detailed examination of this individual revealed that he had sustained extensive injuries two years previously.(11)

Therefore, there was both an in vivo and an in vitro demonstration of the neutralizing effect of the convalescent serum. Isolation of the gamma globulin from convalescent and normal sera revealed that this fraction would likewise neutralize the toxic effect of acute sera, whereas gamma globulin from normal sera failed to do so (Table 3). It was of interest in this regard that the endotoxin from B. coli also inhibited the growth of HeLa cells, but was not neutralized by gamma globulin from convalescent or normal serum.

The children were treated for their burns by standard procedures. The results here reported confine themselves entirely to the effect of the convalescent serum. Because of the limited burn donors available, especially Rh negative blood, only a limited number of transfusions with healed blood or plasma were given. The results to be considered thus will be confined to short periods of time before and after a series of such transfusions. Clear cut results were seen when at least 500 ml of whole blood or 250 ml of plasma were given in a 24-hour period to these children who weighed from 90 to 100 pounds. Although some twelve children had received this type of blood or plasma, only six critically

TABLE 2

EFFECTS OF VARIOUS SERA ON THE GROWTH OF HELA CELL TISSUE CULTURES

	SERUM SOURCE		
	SCHOOL FIRE VICTIMS	NAVY RECRUITS	
		Febrile	Asymptomatic
NUMBER TESTED	16	15	72
NUMBER INHIBITORY	15	0	0

TABLE 3

A COMPARISON OF THE EFFECT OF GAMMA GLOBULIN FROM NORMAL AND SPECIAL DONOR SERUM IN NEUTRALIZATION USED IN COMBINATION WITH PATIENT SERA FOR HELA CELL GROWTH

MICROSCOPIC OBSERVATIONS

PATIENT SERA POOL (ml)	Normal Gamma Globulin ml .2	.1	.05	.01	0	Donor Gamma Globulin ml .2	.1	.05	.01	0
03	--	--	--	--	--	1+	2+	2+	--	--
03	--	--	--	--	ND	1+	2+	1+	--	ND
03	--	--	--	--	ND	1+	1+	±	--	ND
0	1+	ND	ND	ND	2+	1+	ND	ND	ND	2+

ill children received an amount which was considered minimal; and this was administered fourteen to twenty-nine days after burning. The clinical impression was that there was an improvement in all, but in some instances it was described that a "crisis" had occurred within hours after the transfusion (in three subjects who had received more than the minimal amount of blood or plasma). The case history of one such typical patient is described below:[17]

Nine-Year Old Female; Extent of Burn - 46% and Fracture. Condition of child before burned donor transfusions (Dec.14,1958): The child had received up to this point six units of regular blood bank plasma and five units of blood distributed on a daily basis. On Dec. 12 she received one unit of blood and on Dec. 13, two units of plasma. The child was markedly restless and irritable; any change of position would audibly exaggerate the irritability. Interest in her environment was slight; her appetite was poor; she slept only in snatches; movements of extremities were limited and painful. She appeared to be in an extremely toxic condition, with marked edema in the face, neck and extremities (site of burns). Her temperature ranged from 101 to 105°F. (38.3 to 40.6°C.) (Dec.13 and 14).

Transfusion with 400 ml burned donor blood was begun Dec. 14 at 7:10 pm.

Condition of child on Dec. 15 at 11:00am - Nurse and parents were not aware that type of blood of transfusion was different from those given previously. The changes listed below were noted by the parents, the nurse and two physicians who had seen the child before and after the transfusion.

A marked and sudden change for the better was noted. Shortly after the transfusion the child fell asleep and slept for five hours -- this was

the first time she had really slept for days. Her irritability was so reversed that she began to dictate letters for her friends to her father. Her appetite was markedly improved, she had less pain and the spontaneous mobility of her extremities was definitely increased.

On Dec. 15 she received no transfusions of any kind. That night she slept about five hours.

On the morning of Dec. 16 the child was holding her gains well. Toward noon, and increasing with evening, she again became restless and irritable, her appetite slackened, her mobility decreased and the parents were notably disturbed and discouraged. At 8:50 pm. 150 ml of burn blood donor plasma was started and at 10:15 pm the infusion was complete. Soon after the transfusion the child fell asleep and slept all night -- this was the longest time she had slept during her illness. At 10am on Dec.17 the impression of the parents, nurse and/or two physicians was as follows:

The child was better than she had ever been; more alert, more spontaneous movements; less pain; good appetite. What was particularly striking was that the face and neck, which had been markedly swollen, had receded to such an extent that they were of normal size. A decrease in the swelling had been suspected for the previous two days. Her temperature was 101.8°F. (38.8°C.) (rectal).

Dec. 17 - Transfusion with 250 ml of burned donor plasma.

Dec. 18 - All gains held; child ate a fair breakfast of cereal and juice; drinking well; not so difficult a nursing problem; parents in relatively good cheer; neck and face edema still down.

Dec. 18 - Transfusion with 250 ml of burned donor blood.

Dec. 19 - Transfusion with 250 ml of burned donor blood and two units of

blood bank plasma.

Dec. 20 - Transfusion with 250 ml of normal donor whole blood and 150 ml of burned donor plasma.

Dec. 26 - Transfusion with 250 ml of burned donor blood and 250 ml of blood bank plasma. Child doing well; face showed no edema (only exposed part); child has been skin-grafted; mother and father in good cheer (a good sign).

The temperature and selected clinical laboratory studies were plotted; after transfusion with burned donor blood and plasma from Dec. 14 to 20, there was a fall in termperature and urine albumin. Administration of three new antibiotics was initiated from Dec. 13 to 16.

It is true that new antibiotics were constantly being tried in this child as well as in the others and must be considered in evaluating the clinical effects of the healed burn blood or plasma. However, the fact that the antibiotics were never the same in each subject, that in every case clinical effects occured within hours after the administration of the healed burn sera, speaks for a specific effect of the blood or plasma.

More recently a ring precipitin test has been developed which has been applied to acutely burned subjects entering the service at the Cook County Hospital in Chicago. The proficiency of this test, however, depends upon a high titred antiserum for demonstration of "toxin" and highly toxic sera to demonstrate "antibodies." Not all individuals after burning have serologically demonstrable high titred serum; also, it was found that after an acute burn, precipitinogens appeared in highest frequency between forty-nine hours and six days after burning. They were often not found before or after that time, depending upon the nature of the burn. "Precipitins" were detected when healing was far advanced, usually after 35 to

40 days. In the same individual one first noted precipitinogens and later, with healing, "precipitins." Table 4 gives the results of some "precipitin" tests done in subjects with first and second degree burns. It is of interest that in five of these patients followed 35 days or more after burning, none developed"antibodies." On the other hand, in those who had sustained second and third degree burns as seen in Table 5, it is noted that whereas the time of appearance of the "toxin" is about the same, "antibodies" did appear in 58% of the cases. These studies indicate, therefore, that convalescent serum for therapy should be collected from those who have sustained second and third degree burns.

The studies just presented indicate that a "toxin" has been isolated directly from diffusates of burned skins of rats in vivo, circumventing the circulation. In human subjects, this toxic effect was demonstrated in sera of acutely burned subjects by the inhibitory effect on HeLa cells in cultures. Experimentally, in burned rats, "auto-antibodies" are produced which react with the "toxin" isolated from burned skin pocket diffusates. "Antibodies" were produced in the rabbit to this rat pocket "toxin" which partially neutralized the toxic effect of the latter "toxin" in the mouse. In burned human subjects "antibodies" appear as healing progresses, which "antibodies" have a neutralizing effect on the burn "toxin" in vitro (HeLa cells) as well as in vivo (human subjects); the latter was concomitant with clinical improvement. It is suggested, therefore, that convalescent serum of burned human subjects be used in the therapy of acutely burned human subjects. Because the degradation toxic products enter the blood stream as long as large skin surfaces remain exposed, it is necessary to treat acutely burned subjects over the entire time that large open wounds exist, depending of course upon the toxicity of the individual. Such treatment in critically burned subjects

TABLE 4

TIME OF APPEARANCE OF "TOXIN-ANTITOXIN" IN BURNED HUMAN SUBJECTS

(1st and 2nd Degree)

TIME	% BURN	NO. CASES	"PRECIPITINOGENS" NO. POS. CASES	NO. TESTS	NO. POS. TESTS
0-24 hrs.	3-20	6	0	6	0
25-48 hrs.	2-15	11	1	12	1
49 hrs-6 days	3-12	7	2	6	2
7-35 days	3-25	8	0	8	0
			"PRECIPITINS"		
>35 days	3-10	5	0	8	0

TOTAL CASES -- 24. (15 months -63 years)

TABLE 5

TIME OF APPEARANCE OF "TOXIN-ANTITOXIN" IN BURNED HUMAN SUBJECTS

(1st, 2nd, and 3rd Degree)

TIME	% BURN	NO. CASES	"PRECIPITINOGENS" NO. POS. CASES	NO. TESTS	NO. POS. TESTS
0-24 hrs.	3-65	2	0	2	0
25-48 hrs.	2-28	2	0	1	0
49 hrs-6 days	5-65	7	4	7	4
7-35 days	2-10	10	0	12	0
				"PRECIPITINS"	
>35 days	3-65-	33	19	36	19

TOTAL CASES -- 15. (14 months-61 years)

should be given daily; then every other day, every third day, etc., depending upon the severity of the case. This therapy should be continued until less than 10% of the body surface is exposed. Approximately 125 ml of serum or plasma, or 250 ml of whole blood should be given to a 90 to 125 pound child as a minimum daily dose (approximately 1 ml of sera or plasma per pound of body weight). Double that amount was given and is recommended when the situation is desperate. Blood to be collected for use in treatment should be from individuals who sustained second and third degree burns of at least 25% of the body surface. Serologic tests indicate that the best time to obtain this blood is within a year after burning, but sera up to three years gave positive results, and in rare instances, up to five years. Verification of the effectiveness of convalescent sera in burned subjects has been shown by Feodorov[12] in dogs; by Malm[13] in rats; by Millican[14] in mice; and in human subjects by the Russian school headed by Feoderov[15] and in the Czechoslovakian studies of Dobrkovsky et al.[16] There are undoubtedly numerous factors which contribute to the toxic and lethal effects following burning. The fluid, colloid and electrolyte balance; nervous, hormonal and nutritional disturbances and secondary infection, are all important factors. Cognizance of these factors has unquestionably diminished the mortality following thermal injury of moderate severity, but the death rate following severe and extensive burns, still remains high.[18] It is believed that the "toxins" liberated from degradation products of tissue damage have probably injured the reticulo-endothelial system and have rendered such hosts infinitely more susceptible to infection. It is believed that the lowered resistance established by the degradation products of injured tissue make such individuals highly susceptible to organisms not infrequently present on the skin, in the environment or in the nose, pharynx, or hands of personnel ministering to these patients. Further evidence

that the cause of thermal death is not basically one of infection was demonstrated in germ-free animals, where it was shown that the death rate was higher following a thermal insult in germ-free animals than in normal ones.[19] It is proposed that amelioration of the basic injury caused by the burn "toxins" will be necessary before the host can efficiently cope with bacteria or other noxious agents that would invade it. On the basis of present available data, it is believed that one method worthy of further investigation of the effect of such an amelioration is by the use of healed burned donor sera of known in vitro antitoxic potency.

Preliminary animal and human studies on the extraction of toxic substances directly from injured skin and the production of "antibodies" by these degradation products in the homologous or heterologous hosts, suggest the possibility that antisera to these toxic products may be produced artificially.[20] Human skin extracts for active immunization is being explored.[21]

Evidence of the presence of a "toxin" in the blood of acutely burned or injured human subjects has been presented. The existence of "antitoxic-like" substances in the blood of healed burned individuals to the burn "toxin" has likewise been presented. Preliminary clinical evidence suggests a beneficial detoxifying effect of healed burned blood or plasma in critically burned or injured individuals. More clinical trials are recommended, using "titred" antisera of known in vitro potency. Preliminary experimental evidence indicates that it may be possible to produce antisera to "toxic" products of burned or injured skin artificially.

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II. CURRENT PERIOD STUDIES INDICATING THAT TOXIC AND LETHAL SUBSTANCES CAN BE ISOLATED IN VITRO FROM BURNED NORMAL HUMAN SKIN

A. TOXIC AND LETHAL SUBSTANCES FROM BURNED NORMAL HUMAN SKIN

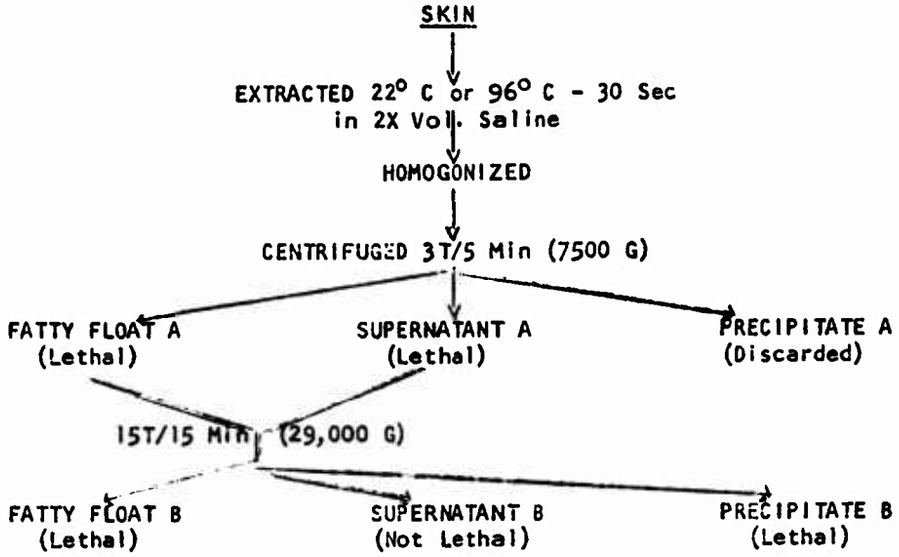
The existence of specific toxins following thermal injury has been denied. Discrepancies have arisen because blood or lymph from burned animals and man have been used for toxin isolation. The adsorbtion, neutralization or breakdown of toxic substances in the blood is well documented and may account for the differences reported. Previous studies from our laboratory, using *in vivo* and *in vitro* preparations for the isolation of the toxins (but in every case circumventing the circulation), have demonstrated that toxins are involved. Said toxins are exclusive of histamine, bradykinin, adenyl compounds, seratonin or salts. Fox of New York reported that the toxic and lethal substances isolated from human skin are in the main to be related to thromboplastin. The following studies, though preliminary, will demonstrate that toxic and lethal substances can be isolated from in vitro burned or not burned normal skin in addition to thromboplastin.

Skin was obtained from post-mortem materials in acute homocidal death, as well as surgical specimens, such as amputation of leg or breast. Specimens were obtained within five hours after death or removal from the body.

Chart I outlines the general method of extraction. The skin was defatted manually and cut up into fine pieces (approximately 3mm cubes). Saline at either room temperature or 96° C was added in the proportion of two volumes of fluid to one volume of skin. After 30 seconds the suspensions were homogenized in a Waring blender (1500 rpm for 1 minute).

CHART I

FLOW SHEET - SKIN EXTRACTION



The suspension was then centrifuged at 3000 rpm for 5 minutes (7500 G) (Crude A). Three layers appeared: a fatty float, a supernatant and a precipitate. In some experiments the fatty float and the supernatant were pooled, extracted or not extracted with acetone (56° C for 1 hour) and centrifuged at 15,000 rpm for 15 minutes (29,000 G) (Crude B). Again a fatty float, a supernatant and a precipitate appeared. The various fractions were used as isolated or were freeze dried and then resuspended in saline. Tests for toxicity or lethality were performed in white Swiss mice 18 to 20 grams. In each case 0.2 ml was injected in the tail vein. The toxic and lethal effects were noted. For this discussion only the lethal effects will be considered. An extract was considered lethal when approximately 50% of the animals died.

Table 6 summarizes the experiments done with the Crude A fractions; that is after 3000 rpm. A comparison is made between the so-called hot (96° C) and cold (room temperature) extracts. It will be noted that a certain degree of lethality was noted for all extracts; but that considering the individual experiments, lethality occurred in 50 and 60% of the experiments for the cold extracts and in 81 and 93% for the hot extracts. The differences between the corresponding hot and cold extracts were significant at the 95 and 97% level respectively. The contention of this laboratory is that any tissue injury, be it physical, thermal or chemical, is associated with degradation products that can be toxic and lethal to the host. Cutting of the skin, homogenization, as was done in these experiments, is a severe form of injury; heat only exaggerated this basic trauma. (See also appendix, experiments 17 & 25)

Considering the milligram of extract to gram of mouse, it was noted that it required less of hot extract to kill than of the cold extract. Table 7 depicts the results after 15,000 rpm (29,000 G). Only the result of

Table 6

LETHALITY OF CRUDE FRACTIONS A (3000 RPM)

Fraction	No. Experiments	No. Animals	% Experiments Lethal	Mg/Gram Animal Lethal
Cold (22° C.) Fatty Float	10	45	60.0	0.175
Hot (96° C.) Fatty Float	28	91	93.0	0.158
Cold (22° C.) Supernatant	10	95	50.0	0.138
Hot (96° C.) Supernatant	32	266	81.0	0.100

the precipitate is given, since the supernatant was not lethal when given in equivalent amounts (up to 0.16 mg per gram mouse). It will be noted that the lethality for the hot precipitate at 15,000 rpm and the hot supernatant at 3000 rpm were similar (0.155 mg per gram and 0.100 mg per gram of animal respectively). Thus these fractions appear to be similar. The fatty float lethality was similar for both Crude A and B fractions. (Approximately 0.2 mg per gram of mouse).

To rule out thromboplastin the following experiments and results are offered: (1) After the homogenization of the skin in hot saline, the homogenates were tested at once, then after two, four and twenty-four hours incubation at 22° C. Toxicity remained more or less constant, as will be noted in Table 8. In one rabbit lung thromboplastin preparation, although highly toxic to the mouse, when this same preparation was allowed to incubate at 22° C for forty-five minutes, the lethality practically disappeared (0.52 mm per gram animal was not lethal);* (2) The various lethal fractions from the Crude A and Crude B preparations were compared before and after heating in activation at 56° for thirty minutes. At higher concentrations there was little to no reduction in the toxicity of these extracts.**

On the other hand the rabbit lung thromboplastin lost at least ten-fold in its toxicity (100% lethal levels) when exposed to 56° C for thirty minutes. (0.3 mg to 2.5 mg per animal); (3) Adding equal parts of the concentrated lethal skin extracts and 10% heparin failed to alter the lethality of the extracts;** (4) Aging of the lethal skin extracts at 5° C for thirty-two days did not alter the lethality;** (5) Ether extraction,** which mainly removes the lipoid component of thromboplastin (active fraction) increased the lethality of the residue (from 0.179 mg per gram

* See Appendix, Experiment 9
**See Appendix, Experiments 25, 26 & 27

Table 7

LETHALITY OF CRUDE FRACTIONS B (15000 RPM)

Fraction	No. Experiments	No. Animals	% Experiments Lethal	Mg/Gram Animal Lethal
Hot Precipitate	7	40	85.7	0.155
Cold Precipitate	2	22	50.0	0.267

Table 8

EFFECT OF INCUBATION (22° C.) ON CRUDE HOMOGENATE

Fraction	No. Experiments	% Experiments Lethal	Mg/Gram/Animal Lethal			
			0 Hrs	2 Hrs	4 Hrs	24 Hrs
Hotty Fatty Float	3	100	0.058	0.137	0.095	0.100
Hot Supernatant	3	100	0.158	0.079	0.131	0.142

to 0.53 mg per gram of mouse).

Attempts at purification and concentration of these toxic substances is now underway. In isolated experiments, for example, it was noted that dialysis against water or saline increased the relative toxicity of the non-dialyzable portions*(Table 9). However, in one experiment where the skin fractions were first extracted with ether and then dialyzed against water (10 parts to 1 for twenty-four hours), three of the four residues lost their toxicity and lethality.**

It is interesting to speculate whether or not the dialyzability of this toxic and lethal fraction is dependent upon its separation or not from a lipoid component.

Toxic and lethal substances have been isolated <u>in vitro</u> from normal, human skin, whether burned or not. Extracts from normal skin were less toxic and lethal than from burned skin. The lethal fractions are apparently heat stable and not affected by heparin or aging in concentration solutions. Extractions with ether increased the toxicity of the residue. A new method is at hand, therefore, for isolation of toxic material from burned, normal skin <u>in vitro</u>.

The significance of this study is that it will be possible to study the burn toxin in greater detail and to determine whether one may use this material as antigen for active and passive immunization and for serologic testing.

* See Appendix, Experiment 17
** See Appendix, Experiment 1

Table 9

EFFECTS OF DIALYSIS ON HOT AND COLD FRACTIONS

Fraction	No. Experiments	% Experiments Lethal	Average Mg/Gram Animal		
			Crude	Dialized HOH	Dialized Saline
Hot Super	3	100	0.157	0.068	0.032
Cold Super	2	50.0	0.147	0.137	0.047

	Animals Injected - % Death		
Hot Super	14 94%	23 91%	18 83%
Cold Super	10 30%	10 20%	10 50%

NOTE:
Saline (NACL) injected 0.525 mg/gram animal was not toxic or lethal to animals

0.740 mg/gram animal was lethal to 31% of animals

B. IMMUNOLOGIC STUDIES IN VITRO

1. Sheep Cell Hemolysins in Sera of Burned Subjects

This is a continuation of studies reported previously. The method is basically one where the sera of acutely burned patients are heated to 60° C for fifteen minutes and then tested for their ability to hemolize sheep cells to which complement has been added.

Twenty-one cases are listed in Table 10 that were tested by the above method and arranged according to the time the blood was drawn after burning. Blood drawn from forty-eight hours to twenty days after burning gave eleven out of twelve positive results. The one case that was negative was a 20% burn. It will be noted that the remainder were from 30 to 95% of body surface burned. Blood drawn twenty-eight to forty-five days afterward yielded one positive case out of nine. This was an extensive third degree burn covering 50% of the body. The titers of the acute sera varied from 1:2 to 1:8, the latter being present in the most extensive burns (45 to 95% of body surface, second and third degree burns).

These studies confirm our earlier ones and indicate that a heat stable hemolysin is thrown into the blood stream for long periods of time, depending upon the extent of the burn.

2. Ring Precipitin Tests in Burned Human Subjects

The method as previously described consists basically of overlaying acute sera in various dilutions onto convalescent serum diluted 1:2 with saline. The acute sera is routinely diluted 1:4 and multiples of this dilution up until 1:64 or more, when titration is indicated. Incubation is at room temperature and readings are made every hour for five hours at least.

As will be noted from Table 11, there were some 431 tests run during the past year. This represented twenty acute patients and seventeen

TABLE 10

SHEEP CELL HEMOLYSIN TEST
AFTER INACTIVATION OF ACUTE BURN SERA AT 60° C FOR 15 MINUTES

SERUM	DEGREE AND PER CENT BURN	TIME POST-BURN	SHEEP CELL HEMOLYSINS	TITER
263	3 (95)	2 days	+	1:8
198	2, 3 (60)	2 days	+	1:4
217	3 (45)	2 days	+	1:8
215	2, 3 (75)	7 days	+	1:4
234	2, 3 (20)	8 days	-	
210	3 (50)	8 days	+	1:4
243	2, 3 (50)	9 days	+	1:2
299	2 (30)	11 days	+	1:2
265	3 (65)	15 days	+	1:8
264	3 (50)	15 days	+	1:4
267	3 (50)	20 days	+	1:4
268	3 (65)	20 days	+	1:8
254	3 (10)	28 days	-	
209	2, 3 (20)	30 days	-	
244	2, 3 (20)	30 days	-	
269	3 (45)	35 days	-	
270	3 (50)	40 days	+	1:2
297	3 (30)	44 days	-	
218	3 (45)	35 days	-	
250	2, 3 (55)	44 days	-	
245	3 (18)	45 days	-	

METHOD OF TESTING: Serum heated at 60° C for 15 minutes. Serial dilutions made with saline. 0.25cc diluted serum, plus 0.1cc of a 2% suspension of sheep cells. Incubated for 1 hour at 37° C. Add 2 drops complement 1:10 plus 0.5cc saline. Incubate 1 more hour at 37° C.

convalescents. Many of these were drawn at different periods of time after their burning and tested when available serum was at hand for the testing. As mentioned previously, since it is not always possible to have a good reacting convalescent serum or a good acute serum at the same time, it makes the results not completely representative of the rate of reactivity.

Considering all tests run, 46% (or 274 tests) were positive when acute sera was overlayed convalescent sera. When acute sera was overlayed normal sera, four out of seventy-eight reacted, or 5%. It was noted, for example, that many convalescent sera would react well when first drawn; but after a month or more of storage (ice box or deep freeze temperatures), the titers would be reduced or negative. Similarly, acute sera when stored lost its ability to react. For individual experiments, see appendix.

Table 12 lists the reactions of acute sera according to the time the blood was drawn after the burn was sustained. It will be noted that reactions occurred more consistently up until twenty-eight days after burning. After this time the results were more or less erratic.

In Table 13 the reactions of human convalescent serum are listed according to the time after burning that blood was drawn. Since it was not always possible to obtain sera from completely healed burned subjects, many of the bloods are from "hospital convalescents;" that is, patients still in the hospital and whose wounds had not completely healed. This accounts for the variability of results, since it is reasoned that as long as there are open wounds there will be antigens poured into the blood stream which will neutralize part or all of the circulating "antibodies."

It is of interest that one sample 330 days after burning was still positive in 64% of tests done with acute sera. It is also of interest that one convalescent sera (298) which gave negative results in five tests against

TABLE II

RING PRECIPITIN TESTS
OF ACUTE AND CONVALESCENT SERA OF BURNED HUMAN SUBJECTS

METHOD	NUMBER TESTS	NUMBER POSITIVE	PER CENT POSITIVE
A/C	274	127	46
N/C	65	0	0
A/N	78	4	5
N/N	14	0	0

A = Acute Burn Serum

C = Convalescent Burn Serum

N = Normal Serum

TABLE 12

RING PRECIPITIN TESTS
OF ACUTE BURN SERA (HUMAN)

SERUM	DEGREE AND PER CENT BURN	TIME POST-BURN	Tests with Convalescent Sera		
			NUMBER TESTS	NUMBER POSITIVE	PER CENT
263	3 (95)	2 days	2	2	100
198	2, 3 (60)	2 days	20	8	40
217	3 (45)	2 days	30	18	60
215	2, 3 (75)	7 days	34	25	73
234	2, 3 (20)	8 days	10	3	30
210	3 (50)	8 days	14	6	42
243	2, 3 (50)	9 days	5	1	20
299	2 (30)	11 days	4	0	0
265	3 (65)	15 days	4	1	25
264	3 (50)	15 days	4	3	74
267	3 (50)	20 days	1	1	100
268	3 (65)	20 days	1	1	100
254	3 (10)	28 days	6	6	100
209	2, 3 (20)	30 days	9	3	33
244	2, 3 (20)	30 days	5	0	0
269	3 (45)	30 days	1	1	100
218	3 (45)	35 days	31	22	70
270	3 (50)	40 days	3	0	0
297	3 (30)	44 days	4	0	0
250	2, 3 (55)	44 days	3	1	30
245	3 (18)	45 days	5	1	20

TABLE 13

RING PRECIPITIN TESTS
OF CONVALESCENT BURN SERA (HUMAN)

SERUM	DEGREE AND PER CENT BURN	TIME POST-BURN	Tests with Acute Sera		
			NUMBER TESTS	NUMBER POSITIVE	PER CENT
233*	2, 3 (15)	30 days	5	0	0
235*	3 (20)	35 days	21	13	62
206*	2, 3 (18)	60 days	20	9	45
249*	3 (20)	60 days	5	1	20
248*	3 (30)	60 days	9	4	44
306*	3 (35)	60 days	11	2	18
247*	3 (45)	60 days	5	3	60
298*	3 (50)	60 days**	5	0	0
197	3 (45)	75 days	32	11	34
212*	2, 3 (18)	90 days	4	2	50
188*	2, 3 (20)	90 days	6	0	0
231*	2 (15)	90 days	9	9	100
272	3 (45)	90 days	14	7	50
216	3 (45)	120 days	24	11	45
213*	3 (20)	150 days	4	1	25
253	3 (30)	180 days	4	2	50
220	3 (50)	330 days	42	27	64

* Hospital Convalescent
** Skin Graft

acute sera was one in which there had been a skin graft formed just before the blood was drawn. Surgery may alter the "antibody" response since during the trauma caused by the skin grafting, neutralization of the "antibody" is a possibility.

In summary, the ring precipitin tests under ideal conditions may be a method of assessing the presence of "toxin-antitoxin" in the blood. However, it is not a consistent finding and depends a great deal upon the potency of both the acute and the convalescent serum, which in turn depends upon the time these bloods are drawn and the extent of the burn. Whether or not these are true antigen-antibody reactions is still to be fully assessed. It will be shown in studies listed below that by some of the other standard techniques for antigen-antibody reactions positive results were obtained with only one additional method (agar double diffusion).

3. Agglutination Tests: Human Skin Extracts-Convalescent Sera

The use of particles in agglutination tests such as colloidin or latex is a well-known procedure. In the hands of Czechoslovakian workers (Pavkova), agglutination of colloidin particles coated with extracts of burned skin was noted when placed in contact with acute or convalescent sera. In the present study a new principle is employed using particles prepared from homogenized human skin (burned in vitro or not burned).

METHODS

a. Preparation of the skin.

The method used was similar to that described for toxicity experiments. The supernatant after 3000 rpm (7,900 g) or the particles sedimented after 15,000 rpm (29,000 g) were used for these tests. These human skins were obtained either at post morten or at surgery, were heated to 96° for thirty seconds or not before homogenization. These materials were toxic and lethal to mice. In addition to using the particles as

mentioned above, in some instances the particles were washed three times in saline. In other instances the particles were dialyzed against distilled water and the residue in the bag was resuspended and used for the test.

b. The Test

In a test tube were added five drops of glycine buffered saline or regular saline; five drops of sera (either concentrated or in seral dilutions) and one drop of particles resuspended in saline to form a heavy suspension. The mixture was shaken and incubated for one hour at room termperature. It was then centrifuged at 100 rpm for three minutes. Tubes were then read for gross agglutination and verified by microscopic examination when indicated.

RESULTS

Particles for the agglutination tests were prepared from nine skins (human material obtained from the morgue or surgery). One extract gave no agglutinations; in three experiments the particles gave similar results with convalescent and normal serum; in five experiments the titers were higher with convalescent serum than with normal serum. In isolated experiments, washing of the particles with saline or dialyzing against distilled water increased their agglutinability. Inactivating the sera at 56° for one-half hour negated its agglutination ability. Agglutination was not restored or enhanced by addition of guinea pig complement. There were 436 tests run, using twelve convalescent sera, sixteen normal sera and five acute sera.

One particular experiment is given in Table 14. There were five convalescent sera and two normal sera compared, using ten different antigens for agglutination. The best results were obtained with the burned skin sediment after 15,000 rpm; these particles, washed three times with

TABLE 14

HUMAN SKIN PARTICLE AGGLUTINATION TESTS

PARTICLES	272(C) TITRE	295(C) TITRE	220(C) TITRE	283(C) TITRE	197(C) TITRE	289(N) TITRE	284(N) TITRE
(a)	2+ 1:4	2+ 1:4	1+ 1:4	1+ 1:4	2+ 1:4	1+ 1:2	1+ 1:4
(b)	-	-	-	-	-	-	-
(c)	2+ 1:8	2+ 1:8	2+ 1:8	2+1:16	2+ 1:4	1+ 1:4	1+ 1:4
(d)	1+ 1:4	2+ 1:4	2+ 1:4	2+ 1:8	2+ 1:2	1+ 1:2	1+ 1:2
(e)	2+1:16	2+ 1:8	2+1:16	2+1:16	2+ 1:4	2+ 1:4	2+ 1:4
(f)	2+ 1:4	2+ 1:4	2+ 1:4	2+ 1:8	1+ 1:4	1+ 1:2	1+ 1:2
(g)	3+1:32	3+1:16	3+1:16	3+1:64	3+ 1:8	3+ 1:8	2+ 1:8
(h)	2+ 1:4	2+ 1:4	1+ 1:4	2+ 1:8	1+ 1:4	1+ 1:4	1+ 1:2
(i)	3+1:32	3+1:16	3+1:32	3+1:32	3+ 1:8	3+ 1:8	3+ 1:8
(j)	3+ 1:4	3+ 1:4	2+ 1:4	2+ 1:4	3+ 1:8	3+ 1:4	3+ 1:4

(C) = Convalescent Sera
(N) = Normal Sera

Particles prepared from surgical specimen

(a) Hot supernatant after 3,000 rpm
(b) Cold particles from supernatant after 3,000 rpm
(c) Hot sediment after 15,000 rpm
(d) Cold particles sediment after 15,000 rpm
(e) Particles (c) washed 3 times with saline
(f) Particles (d) washed 3 times with saline
(g) Hot supernatant (a) dialized against distilled water
(h) Cold supernatant (b) dialized against distilled water
(i) Particles (c) dialized against distilled water
(j) Particles (d) dialized against distilled water

saline; the hot supernatant after 3000 rpm dialized against distilled water; or the sediment after burning and 15,000 rpm. In each case there was a higher titer for the convalescent sera than for normal sera. The non-burned sediment, although showing agglutination, did not yield higher titers for convalescent sera than for normal sera.

These are only preliminary results of an interesting phenomenon which is being pursued further. No definite conclusion can be drawn at this time.

C. COMPARISON BETWEEN THE RING PRECIPITIN TESTS AND OTHER IMMUNOSEROLOGIC TESTS

METHODS

1. Agar Diffusion According to Ouchterlony[1] in Agar Plates

Eleven acute and eight convalescent sera were tested, using wells containing convalescent and others containing normal or acute sera (1% agar in buffered saline m/100 phosphate at ph 7.2). There were no reactors noted between acute and convalescent sera in any of these specimens. Using anti-human serum from rabbits against human serum gave typical lines.

2. Double Diffusion Tests (Agar tube technique[13])

The underlay was 1:2 diluted convalescent sera in a 2% agar suspension. The middle layer consisted of 1% agar in saline and the overlay consisted of a 1:4 dilution of acute sera. In some experiments the acute and convalescent sera layers were reversed. In other experiments normal sera replaced either the acute or convalescent sera. Readings were made after forty-eight hours and longer (Table 15). It will be noted that of fifteen tests on three convalescent sera and eleven acute sera, eleven gave positive reaction. In one convalescent serum all five tests were positive. Using normal sera to overlay the three convalescent sera in six tests, all but one were negative. Using four acute sera with three convalescent sera in twelve tests, eight tests were positive. In four

TABLE 15

AGAR DIFFUSION (TUBE METHOD)

SERA 1:2 DILUTED IN 2% AGAR AS UNDERLAY	ACUTE SERA 1:4 DILUTED AS OVERLAY			CONVALESCENT SERA 1:4 DILUTED AS OVERLAY			NORMAL SERA 1:4 DILUTED AS OVERLAY		
	POS	NEG	TOTAL	POS	NEG	TOTAL	POS	NEG	TOTAL
CONVAL. 1	3	2	5	-	-	-	1	1	2
CONVAL. 2	5	0	5	-	-	-	0	2	2
CONVAL. 3	3	2	5	-	-	-	0	2	2
ACUTE 1	-	-	-	2	1	3	0	1	1
ACUTE 2	-	-	-	2	1	3	0	1	1
ACUTE 3	-	-	-	2	1	3	0	1	1
ACUTE 4	-	-	-	2	1	3	0	1	1
NORMAL 1	1	4	5	-	-	-	0	2	2
NORMAL 2	-	-	-	2	1	3	0	1	1

normals used as overlay instead of the acute sera, none were positive. When acute sera was overlayed normal sera, one of the five gave a positive reaction. When normal sera was overlayed normal sera, none were positive. The line of reactivity when it occurred was usually in the middle (1% agar) layer, very close to the overlay. This occurred when acute or convalescent sera was used as overlay or underlay. The exact nature of this reaction will be investigated further.

According to the best authorities, there would be no inconsistency if a positive precipitin line occurred in a one-way system as in the tube system and not in a multiple way system, as in the plate system of Ouchterlony (see monograph by Lind).

3. Immune Electrophoresis

Electrophoresing acute serum in 1% agar in barbital buffer (iron concentration gamma = 0.024) and then adding convalescent sera in the trough along side of it, according to the method of Grabar and Scheidegger, resulted in no precipitation bands between the acute and convalescent sera.

4. Precipitin Reaction on Cellulose Acetate Paper Impregnated with Convalescent or Acute Sera According to Feinberg[3]

Cellulose acetate paper was immersed in undiluted and 1:3 dilutions of convalescent sera in buffered saline. After blotting to remove excess liquid, the antigen solutions were spotted in microdrops on the damp paper, using a micropipette. The strips were incubated in a moist chamber or under mineral oil for several hours (usually overnight). The papers were then washed in buffered saline and stained with Ponceaus or nigrosin. For controls the strips were dipped in rabbit anti-human sera 1:10 in buffered saline (m/100 normal phosphate ph7). No definite precipitation line bands were noted whereas definite bands were noted when the anti-human sera was tested against human sera.

5. Immunodiffusion on Cellulose Acetate Paper

The method is similar to that of Ouchterlony except that cellulose acetate paper is used. The technique was similar to that described above. There was/no precipitation noted between acute and convalescent sera, whereas sharp lines were noted when rabbit antisera was tested against the human sera as above.

6. Quantitative Precipitation Between Acute and Convalescent Sera[4,5]

The microprecipitin technique of Maurer et al was used, wherein antigen and antibody are mixed for several days in the ice box and the precipitates gathered and the nitrogen contact determined. The same samples were first centrifuged for twenty minutes at 7500 g and then were made up in the same dilutions as used for the ring precipitin tests; thus:

a. acute sera 1:4 dilution with saline

b. convalescent sera 1:2 dilution with saline

(1) 0.5 ml of (a) were mixed with 0.5 ml of (b)

(2) 0.2 ml of (a) were mixed with 0.8 ml of (b)

(3) 0.8 ml of (a) were mixed with 0.2 ml of (b)

(4) 1.0 ml of (a) as a control

(5) 1.0 ml of (b) as a control

The experiments were done with two different convalescent sera and two different sera from acute burned patients who gave a positive reaction in the ring precipitin test. After two, three, six and eight days storage in the icebox in one experiment and after two and nine days in the other experiment all tubes were centrifuged twenty minutes at 7500 g and the supernatants were carefully removed. There were no visible precipitates. The tubes were filled with five ml saline and centrifuged twenty minutes at 7500 g. This washing procedure was repeated once more, then the microportein determination according to Lowry[5] with Folin-

Ciocalteu reagent was used. In all six experiments a variation of the values between 7.5 and 12.7 microgram protein was observed, but no significant difference between the mixtures and the diluted sera alone were noted (Table 16). The control experiment was done with rabbit anti human serum and human serum. Dulutions of human serum from 0.76 microgram/0.1 ml to 76 microgram/0.1 ml in 0.9% saline (buffered with m/30 phosphate and m/15 phosphate, ph 7.4) were mixed with 1 ml of anti human serum dilution 1:5 and stored over night in the icebox. Identical precipitation curves were obtained, using different concentrations of phosphate (Table 17).

Under the conditions of these experiments no precipitate was obtained in several mixtures of acute and convalescent serum.

In summarizing then, it appears that precipitation lines do occur when acute and convalescent sera are in opposition either in the saline ring precipitin tests or in the double diffusion agar precipitation tests. In the latter there is no contact directly between acute sera and convalescent sera, and therefore it cannot be a matter of a physical change occurring when the two sera are in contact with each other. That reaction lines are not noted by the Ouchterlony or immune electrophoresis (Grabar) techniques might be explained, as already mentioned above, by the fact that low-reacting sera diffusion as occurs in these two tests may negate the reaction or make it invisible. The same explanation might be offered for the cellulose acetate paper technique and the qualitative precipitation reactions. However, these are not normal, typical reactions as one usually sees. It should be borne in mind, however, that the skin is a very poor antibody builder and one often sees local reaction where they cannot detect it serologically in skin condition (the so-called atopins and reagins of the allergist). Further studies will have to be made to clarify this atypical phenomenon.

TABLE 16

PRECIPITATION BETWEEN HUMAN CONVALESCENT AND ACUTE BURN SERA

(a) 264(A) Against 272(C)

	Days in the Icebox			
	2	3	6	8
Experiment #	Microgram of Protein	Microgram of Protein	Microgram of Protein	Microgram of Protein
1	9.2	11.9	9.6	-
2	10.2	11.7	12.3	8.4
3	8.2	10.4	8.7	-
4	8.8	8.6	8.8	8.5
5	-	12.7	10.1	8.1

(b) 294(A) Against 272(C)

1	9.0	-	-	9.2
2	10.0	-	-	8.3
3	9.2	-	-	7.5
4	-	-	-	8.0
5	7.8	-	-	6.5

(A) = Acute
(C) = Convalescent

TABLE 17

ANTIGEN - ANTIBODY
PRECIPITATION BETWEEN HUMAN SERUM
AND RABBIT ANTIHUMAN SERUM

MICROGRAM SERUM PROTEIN (TOTAL HUMAN SERUM)	MICROGRAM ANTIGEN-ANTIBODY PRECIPITATE in 0.9% NaCl/M/30 PHOSPHATE PH 7.4	MICROGRAM ANTIGEN-ANTIBODY PRECIPITATE in 0.9% NaCl/M/15 PHOSPHATE PH 7.4
76	-	-
7.6	76	69
3.8	44	42
2.5	28	27
1.5	19	17
0.76	15	11

III. CURRENT PERIOD STUDIES ON STANDARD BURN

A. BASKET TECHNIQUE FOR PRODUCING STANDARD THERMAL INJURY IN RATS

In the study of various aspects of burning, it has been difficult to separate the effects of tissue breakdown due to the burning per se and the secondary infection which invariably sets in. It is important both for basic considerations and for therapy to separate these two phenomena; likewise it is important to have an experimental preparation for inducing thermal injury in animals which is repeatable.

The following experimental method satisfies the above two requirements: that is it minimizes secondary infection and is reproducible. Previously the basket technique for producing a standard scalding burn was described for mice. This report considers rats. As for mice, the affects of anesthesia, weight, temperature of water and time of burning on the LD50 were studied.

Stainless steel baskets (64 mesh) were constructed so that the rats fitted into them snugly and were secured firmly by a lid which covered the chest and abdomen only. The basket was shaped so that the bottom accepted the curvature of the back of the animal, and the ends were tilted upward so that the urethal and anal orifices, the tail and the head were at a level above the anterior axillary line of the rat. Lateral stainless steel arms projected from the basket in such a manner and position that when the preparation was set into a water bath it remained fixed and the entire back of the animal up to the posterior axillary line was submerged in water. The bath was so constructed that it received the basket and the remaining surface of the water was covered. (See previous study).

Two sizes of the basket were used; one to receive an 80 to 125 gram rat; the other to receive a 200 to 220 gram animal.

PERIOD OF ACCLIMATIZATION

The animals were allowed to remain in their permanent quarters for at least one week before they were used for these experiments. The increase in body weight in one experiment was from 126 to 270 grams at the end of three weeks. At one week there was a gain of 70 grams, in the second week 45 grams and in the third week 25 grams.

CHOICE OF ANESTHESIA

SODIUM NEMBUTAL

Sodium Nembutal was injected intraperitoneally. As will be noted in Table 18, the LD50 due to Nembutal (100 to 125 gram rat) is 5.8×10^{-3} mgs per gram of animal at twenty-four hours. To obtain complete relaxation, 3×10^{-2} mgs per gram of rat was needed. Animals showed a great variability due to the affects of Nembutal. As will be seen in Table 19, rats burned at 80° C (100 to 125 gram animals) under Nembutal anesthesia followed an irregular pattern of rate of mortality up to twenty-five seconds of burning.

ETHER

Animals were placed in a desicator with cotton-soaked ether at the bottom but not in contact with the animal. Complete relaxation of the animal was noted in about one or two minutes. Usually the respiration was slowed and regular. At this point the animal was ready for scalding. From Table 20 it will be noted that the LD50 for rats (100 to 125 grams) which were burned at 80° corresponded to the animals in weight and time as given for Nembutal anesthesia above. It will be noted that there was a direct relationship between the time of burning and the death of the animals at twenty-four and forty-eight hours. Because of the regularity of results and because ether anesthesia is of relatively short duration, this anesthesia is considered the one of choice.

TABLE 18

EFFECT OF Na-NEMBUTAL ON
100-125 GRAM RATS

MG NEMBUTAL / GRAM ANIMAL	% DEATH 24 HRS	% DEATH 48 HRS
2.3×10^{-3}	0% 5 - 0	0% 5 - 0
5.6×10^{-3}	40% 5 - 2	40% 5 - 2
8.0×10^{-3}	100% 5 - 5	100% 5 - 5

TABLE 19

(Experiment 1 - 4-4-62)

100-125 GRAM ANIMALS UNDER
Na NEMBUTAL - BURNED AT 80° C

SEC. BURNED	% DEATH 24 HRS	% DEATH 48 HRS
5	0 6-0	0 6-0
10	0 6-0	0 6-0
15	16 6-1	16 6-1
20	33 6-2	33 6-2
25	16 6-1	16 6-1
30	100 5-5	100 5-5
40	100 5-5	100 5-5
50	100 5-5	100 5-5
60	100 5-5	100 5-5
CONTROL	9 11-1	9 11-1

RELATIONSHIP OF WEIGHT OF ANIMAL, ANESTHESIA AND TEMPERATURE OF WATER TO THE LD50 FOLLOWING SCALDING

Tables 20, 21, 22 and 23 depict the mortality forty-eight hours after burning at various weight levels of the rats and temperatures of water. By charting these results, one may interpolate the LD50.

Comparing Nembutal to ether anesthesia for 100 to 125 gram animals burned at 80° C (±1°C): for ether the LD50 was 19.2 seconds and for Nembutal 22.8 seconds.

Comparing the 80 to 90 gram animal to 100 to 125 gram animal under ether anesthesia (80° C): the LD50 for the lower weight animal was 10.8 seconds; whereas for the larger animal it was 19.2 seconds.

Comparing differences in temperature (that is 80° to 90° C (± 1°C), it was noted that the LD50 at 80° was 10.8 seconds; whereas at 90° it was 8.4 seconds (80-100 gram rats).

Comparing 80 to 100 gram animals to 200 to 220 gram animals burned at 90° C, it was noted that the LD50 was 8.4 seconds for the smaller animals and 13.4 seconds for the larger animals.

SUMMARY

It may be summarized that under given conditions repeatable results can be expected by using the basket technique. However, variations are noted between animals given Nembutal anesthesia and ether anesthesia. There is an inverse relationship between mortality due to scalding and weight of animal, but a direct relationship between mortality and temperature of scalding.

TABLE 20
(Experiment 2 - 5-16-62;5-23-62)

100-125 GRAM ANIMALS UNDER
ETHER - BURNED AT 80° C

SEC. BURNED	% DEATH 24 HRS	% DEATH 48 HRS
15	23 13-3	30 13-4
20	46 13-6	61 13-8
25	77 13-10	84 13-11
30	100 13-13	100 13-13
CONTROL	0 13-0	0 13-0

TABLE 21
(Experiment 3 - 5-25-62;6-4-62;6-14-62)

80-90 GRAM ANIMALS UNDER
ETHER - BURNED AT 80° C

5	0 18-0	0 18-0
10	50 20-10	50 20-10
13	67 15-10	67 15-10
15	83 18-15	83 18-15
CONTROL	0 19-0	0 19-0

TABLE 22
(Experiment 4 - 1-7-63; 1-8-63)

80-100 GRAM ANIMALS UNDER
ETHER - BURNED AT 90° C

SEC. BURNED	% DEATH 24 HRS	% DEATH 48 HRS
5	0 18- 0	0 18- 0
8	40 20-8	40 20-8
10	72 18-13	83 18-15
13	100 18-18	100 18-18
15	100 8- 8	100 8- 8
CONTROL	4 23-1	4 23-1

TABLE 23
(Experiment 5 - 1-11-63; 1-14-63)

200-220 GRAM ANIMALS UNDER
ETHER - BURNED AT 90° C

8	0 20-0	0 20-0
12	15 20-3	25 20-5
15	70 10-7	70 10-7
16	55 11-6	81 11-9
18	80 10-8	80 10-8
20	100 10-10	100 10-10
CONTROL	0 20-0	0 20-0

B. STUDIES ON SERUM PROTEINS IN RATS FOLLOWING SCALDING WITH THE STANDARD BASKET TECHNIQUE

These studies were done in cooperation with the protein laboratory of Dr. G. Schumacher, University of Tuebingen, West Germany Department of Gynecology and Obstetrics with assistance from M. Feifel and H.D. Schlumberger.

A former research program, supported by the "Deutsche Forschungsgemeinschaft" Schumacher et al (University of Tuebingen), studied serum protein changes in inflammation and following tissue trauma. The courses of the electrophoretical fractions, of C-reactive Protein, Properdin and complement were observed.[10,11]

Special interest was directed toward alpha$_2$-globulins. The alpha$_2$-globulin fraction has a heterogeneous composition. The main components are haptoglobin, ceruloplasmin, alpha$_2$-macroglobulin and alpha$_2$-lipoprotein. Several other components in lower concentrations migrate in the alpha$_2$-globulin fraction also, such as alpha$_2$-HS-mucoid (identical with Ba-alpha$_2$-glycoprotein (Schmid) and alpha$_2$-Z-globulin (Heremans), alpha$_2$-Neuraminoglycoprotein (Schultze) and a few enzymes and blood coagulation factors.

In clinical studies following operations it was noted that the increase of alpha$_2$-globulins was caused by a very high increase of haptoglobin. The ceruloplasmin also increased slightly. In animal experiments in rabbits, the findings were very similar. The model experiment in rabbits, which were injected with sterile muscle homogenate from rabbits, showed a high increase of alpha$_2$-globulin, haptoglobin and ceruloplasmin. It was the aim of the following experiments to show whether the alpha$_2$-globulins, haptoglobin and ceruloplasmin change after burning or scalding.

1. Experiments with Rats

Animals and Method of Scalding:

White sprague-Dowley rats with a body weight between 150 grams and 22 grams (6 to 8 weeks old) were scalded with a standard technique according to Dr. S. R. Rosenthal.[6] The rats were anesthesized with ether in a small dosage, just enough for a complete relaxation for two to three minutes. The rats were fixed in a basked of stainless steel and plunged for seven seconds in a waterbath of 95° (±1°) C. The basket permitted the back of the rat to be scalded in an exactly reproduceable manner. Around 30% of the body surface was scalded. (In separate experiments with this method the LD50 for rats in this range of body weight was determined to be between twelve and fourteen seconds).

Blood Samples:

Following scalding, blood was drawn from ten groups of rats, eight to twelve rats in every group.

a. After two hours
b. After five hours
c. After twenty-four hours
d. After forty-eight hours
e. After three days
f. After five days
g. After ten days
h. After twenty days
i. After forty days
j. After three months

Blood was drawn by heart puncture without anesthesia. The samples were centrifuged one-half to one hour later, the serum was sealed in ampules and stores in a deep freezer at -18° C.

Methods:

a) Haptoglobin - Determination of hemoglobin binding capacity (HbBC) according to Connel and Smithies[7] using a human hemoglobin preparation and a standard curve for the peroxydase/HbBC calculation.[8]

b) Ceruloplasmin determination according to Broman,[9] using the oxydase reaction with p-phenylendiamine.

c) Alpha$_2$-macroglobin and alpha$_2$ lipoprotein determination by immuno-precipitation[4,5] with specific antisera (Behringwerke, Marburg, West Germany).

d) Paper electrophoresis with the Beckman/Spinco equipment, using barbital buffer, PH 8.6 and the automatic recorder "Analytrol." The total protein determination was made by the copper sulfate method.

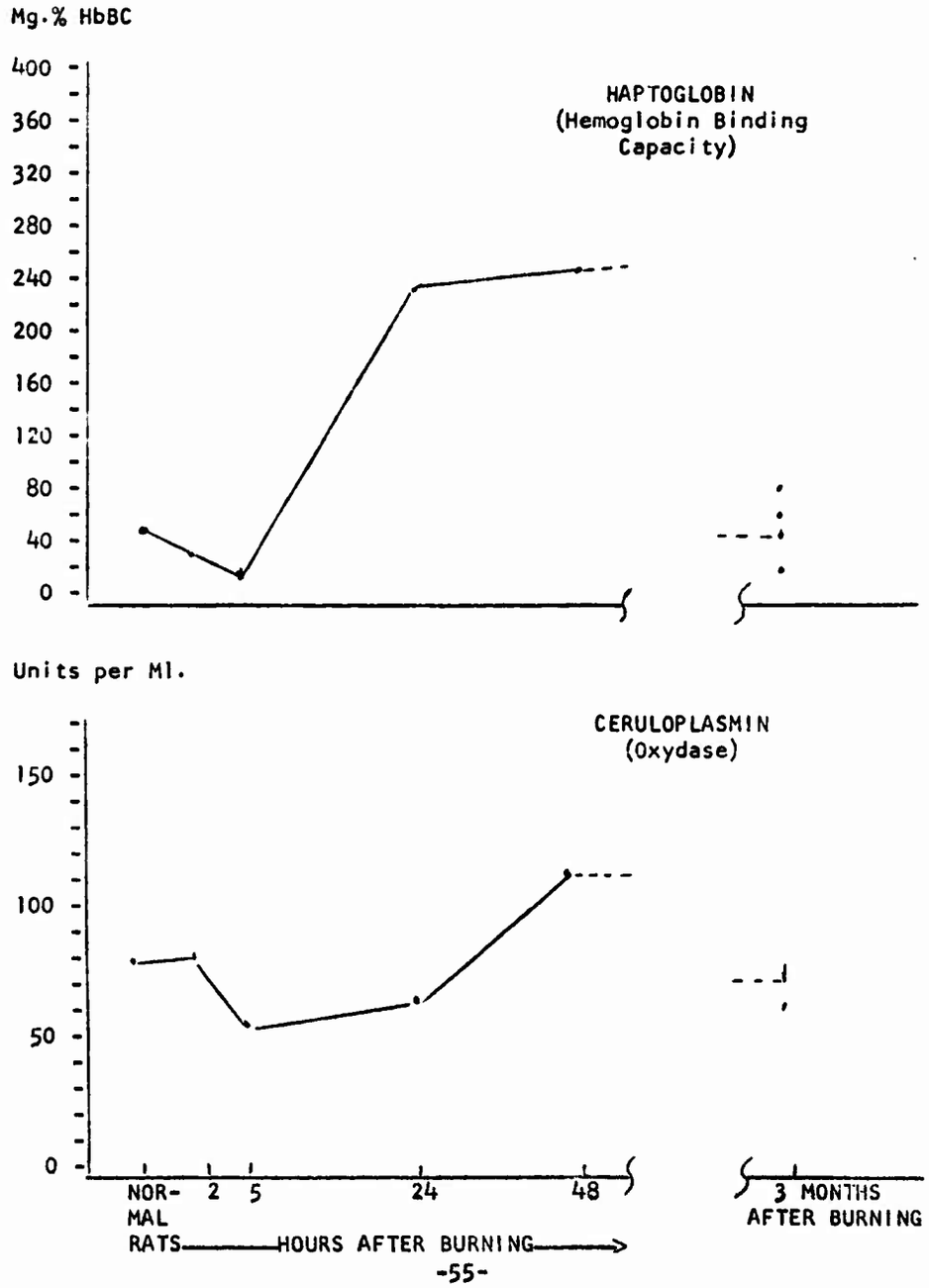
Observations:

The experiments are not yet completed, but the serum values of the first two days after burning are available together with an incomplete group of rat sera three months after burning (convalescent serum).

In the first five hours after scalding, the electrophoretical pattern demonstrated that there was a slight increase or decrease of the alpha$_2$-, alpha$_2$-beta and the beta fraction. At the same time albumin, alpha$_1$- and gamma-globulin decreased. Hemoconcentration changes in the capillary permeability and disturbance of kidney functions may be involved at this time. Hemolysis and hematuria were observed in the first hours. Haptoglobin and ceruloplasmin decrease at this time. The five-hour values are statistically significantly lower than normal. The haptoglobin decrease may be caused by processes of hemolysis. It is not clear if the decrease of ceruloplasmin is related to these or other processes. The changes after twenty-four hours and forty-eight hours

GRAPH 1

SERUM HAPTOGLOBIN AND CERULOPLASMIN
FROM RAT SERA POST SCALD (BASKET TECHNIQUE)



are more marked. The albumin shows an increase. Alpha$_1$-globulin is not changed. The alpha$_2$-globulins increase to statistically significant values after twenty-four hours. An increase of alpha$_2$/beta- and beta globulins is observed only after forty-eight hours.

The gamma globulins decrease continuously in the first twenty-four hours and are still low after forty-eight hours. The difference between the normal values and the values after forty-eight hours is statistically significant.

The preliminary studies on a few sera of convalescent rats three months after scalding (completely healed) show highly elevated gamma-globulins and slightly elevated alpha$_1$-, alpha$_2$- and alpha$_2$/beta globulins.

Graph 1 shows the changes in the haptoglobin- and ceruloplasmin levels. Whereas the ceruloplasmin is low during the first twenty-four hours, the haptoglobin is increased between the fifth and twenty-fourth hour. The increase of ceruloplasmin is about 50% and the increase of haptoglobin is about 400% of the normal values. Both changes are statistically significant. In serum of convalescent rats three months after scalding haptoglobin and ceruloplasmin values are normal.

SUMMARY

The rat experiments are not completed at this time. These preliminary findings indicate that scalding of rat skin causes a sizeable increase of alpha$_2$- and beta-globulins and a decrease of albumin and gamma globulin. Within forty-eight hours after an initial decrease, haptoglobin and ceruloplasmin as components of the alpha globulin group increase. The most remarkable increase occurs in the haptoglobin. The 400% increase which was observed, is approximately in the same range as the increase in human sera after serious operations or in rabbit sera after intramusculary injection of large amounts of sterile

muscle homogenate. The experiments show that tissue breakdown processes induce a rapid increase of haptoglobin in the serum. The haptoglobin obviously has an important significance in the relationship between tissue trauma and the response of the organism. Further experiments are in progress to determine if there is a biochemical relationship between tissue breakdown processes and haptoglobin.

2. Preliminary Clinical Studies

In a few pilot experiments sera from acutely burned patients and from convalescent patients were investigated in a similar manner as in previous clinical postoperative studies. Electrophoresis and a complete analysis of the alpha$_2$-globulins (haptoglobin, ceruloplasmin, alpha$_2$-macroglobulin, alpha$_2$-lipoprotein) were performed. Nine sera from acute burned patients and four sera from convalescent patients were tested. The percentage of the burned body surface varied between 10% and 90% and the degree of burn also varied (second and third degree). The preliminary findings indicate that the albumin is low, the alpha$_1$- and alpha$_2$-globulin values are highly elevated, the values of the beta-globulins are normal and the gamma-globulins are moderately elevated. The values of the electrophoretic fractions of convalescent sera are in the normal range, except highly elevated gamma-globulins (100% to 200%).

The analysis of the alpha$_2$-globulins of the acute cases indicates a high increase in haptoglobin, but low values for ceruloplasmin, alpha$_2$-macroglobulin and alpha$_2$-lipoprotein. The residual part of the alpha$_2$-globulins seems to be significantly elevated.

The analysis of the alpha$_2$-globulins of the convalescent cases indicates a normal or slightly elevated haptoglobin level, still lower ceruloplasmin values, normal alpha$_2$-macroglobulin and alpha$_2$-lipoprotein values,

and a small amount of the undetermined alpha$_2$-globulins. The small number of cases does not allow one to draw any definite conclusions, but the haptoglobin seems to play a role in burned patients. Surprisingly, the ceruloplasmin is low, possibly in connection with liver malfunction. It may be that the highly elevated gamma-globulins are related to liver malfunction as well. But the main reason for the gamma-globulin increase is probably an increased antibody production. Whether this is due to bacterial infection of tissue breakdown products is yet to be determined. Similarly the values noted in acutely burned cases may be influenced by bacterial infection, antibiotics, etc.

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IV. FUTURE STUDIES

1. To isolate toxic and lethal products from burned normal human skin.
2. To determine whether such products inoculated for active and passive immunization will protect against the toxic effects of thermal injury.
3. To develop serologic methods of titering acute and convalescent sera of burned human subjects employing the products of (1) and (2).
4. A double blind control study comparing convalescent serum from burned human subjects and normal human serum in the treatment of acutely burned human subjects.
5. To determine the role of convalescent serum from burned human subjects on the tolerance or rejection of homologous skin grafts.

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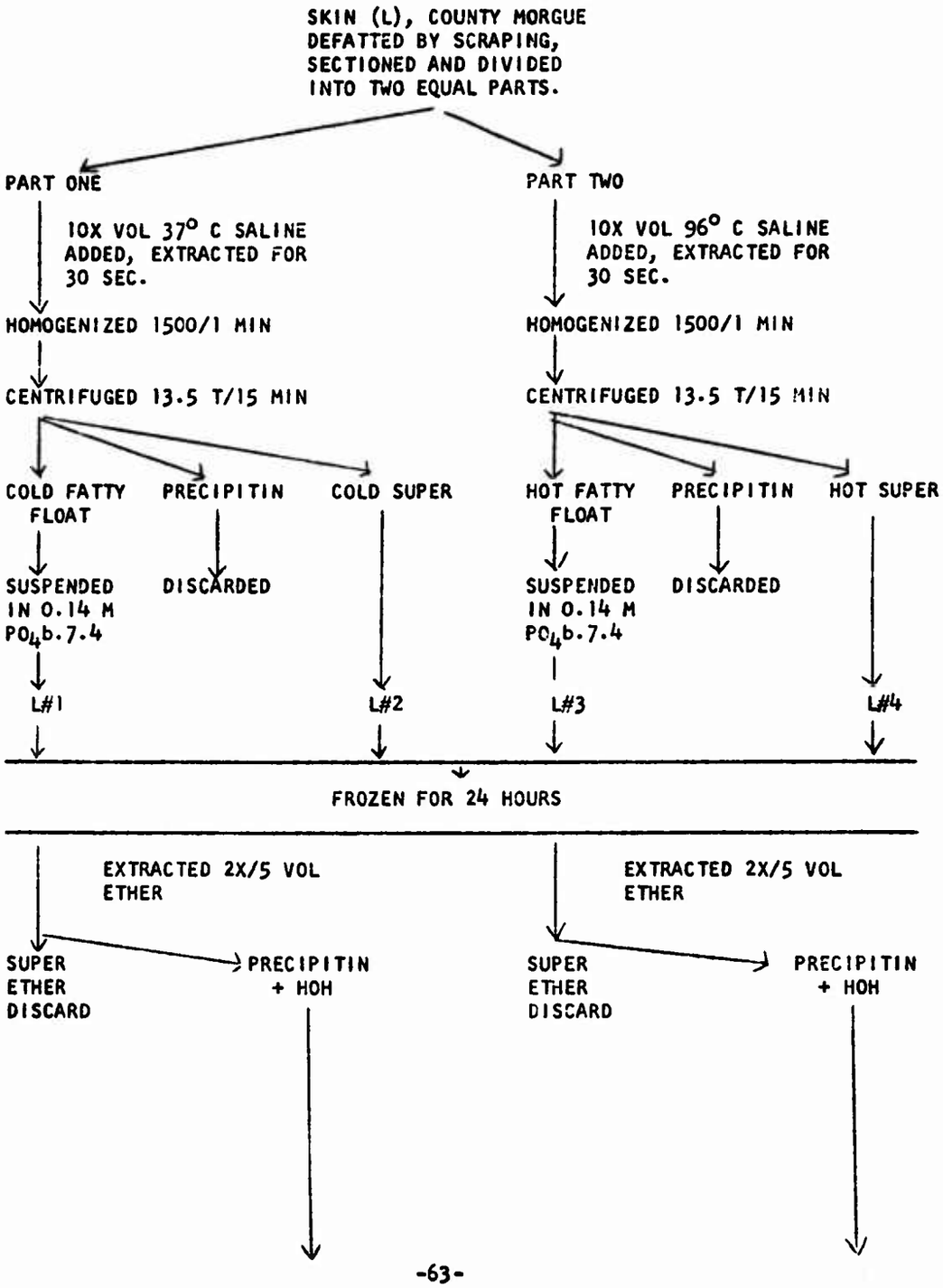
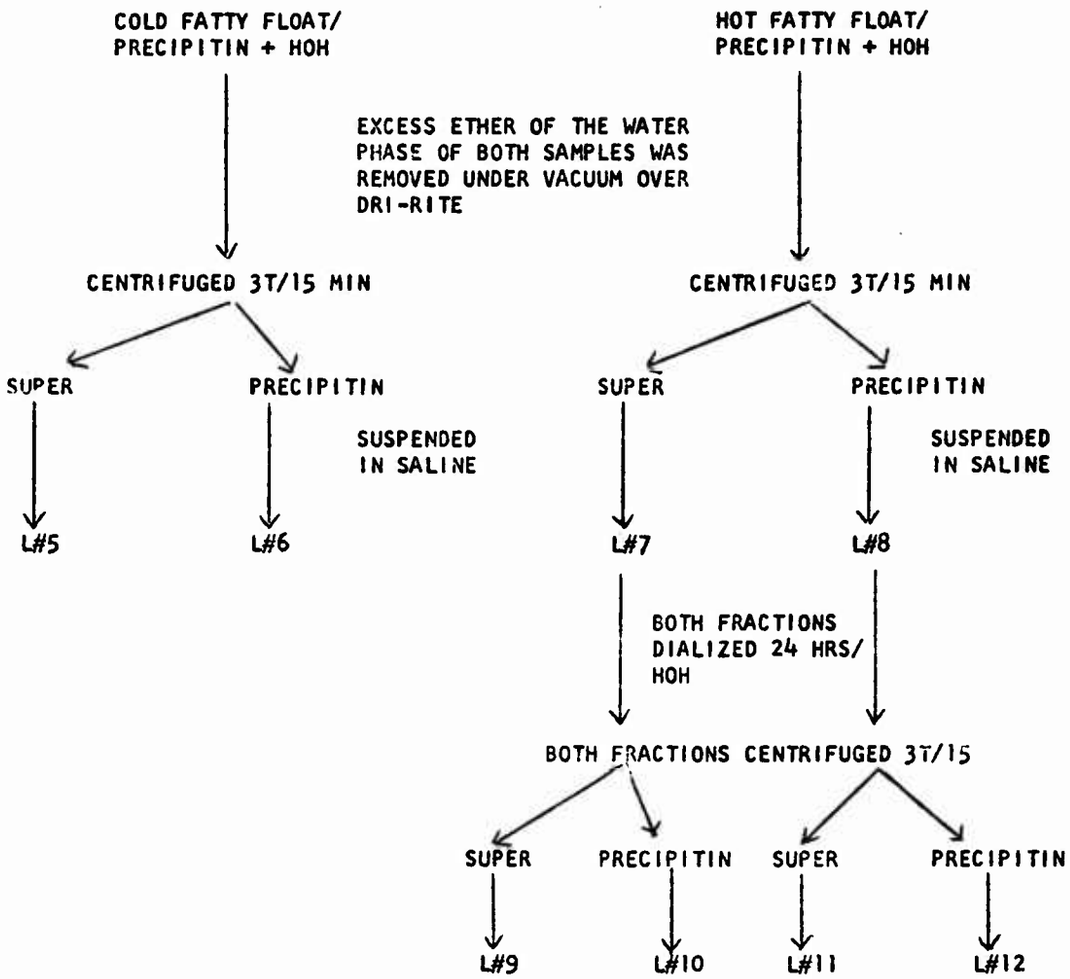
VI. APPENDIX A

EXPERIMENT I



SKIN (L) FRACTIONATION (CONTINUED)



NOTE: PRECIPITINS WERE SUSPENDED IN 0.9% SALINE

TOXICITY RESULTS ON NEXT PAGE

RESULTS EXPERIMENT ONE 6-23-62

SAMPLE	TOXICITY IV	AMOUNT INJECTED IN MLS.	# ANIMALS	TOXICITY IC	AMOUNT INJECTED IN MLS.	# ANIMALS
L#1	SLIGHT REACTION	0.2	0/2	LETHAL	0.03	2/2
L#2	NO REACTION	"	"	SLIGHT REACTION	"	0/2
L#3	SLIGHT REACTION	"	"	LETHAL	"	2/2
L#4	NO REACTION	"	"	SLIGHT REACTION	"	0/2
L#5	LETHAL	0.25	1/1	**********		
L#6	**********					
L#7	LETHAL	0.25	2/2	LETHAL	0.03	2/2
L#8	LETHAL	0.25	2/2	**********		
L#9	NO REACTION	0.25	0/2	**********		
L#10	NO REACTION	"	0/2	**********		
L#11	NO REACTION	"	0/2	**********		
L#12	LETHAL	"	2/2	**********		
L#8 1/2	LETHAL	0.15	1/1	**********		

NOTES: L#6 lost in centrifugation.
***** = not tested
Inactivation of L#7 and L#8 at 56 C for 30 mins. did not affect toxicity

EXPERIMENT 3 7-3-62

SKIN (N), R+E 539601 AMP DEFATTED BY SCRAPING, SECTIONED AND SUSPENDED IN 2X VOL 96° C SALINE FOR 30 SEC. HOMOGENIZED 1500/1 MIN. AND CENTRIFUGED 3T/15 MIN.

SUPER N#1

HOT FATTY FLOAT N#2

PRECIPITIN DISCARDED

N#2 WAS SUSPENDED IN 0.14 M PO_4 b

INACTIVATED 62° C/30 MIN N#8

EXTRACTED WITH ETHER, (ETHER DISCARDED) PRECIPITIN DISSOLVED IN SALINE N#9

INACTIVATED 62° C/30 MIN N#3

EXTRACTED WITH ETHER, (ETHER DISCARDED) PRECIPITIN DISSOLVED IN SALINE N#4

EXTRACTED WITH ETOH (ETOH DISCARDED) PRECIPITIN DISSOLVED IN SALINE N#5

EXTRACTED WITH ETHER (ETHER REMOVED), EXTRACTED WITH ETOH (ETOH REMOVED) PRECIPITIN DISCARDED AND ETOH FRACTION DRIED IN VACUO. DISSOLVED IN SALINE N#6

ETHER SUPER FROM N#4 AND ETOH SUPER FROM N#5 COMBINED. DRIED IN VACUO AND DISSOLVED IN SALINE N#7

TOXICITY DATA ON FOLLOWING PAGE

RESULTS EXPERIMENT THREE 7-3-62

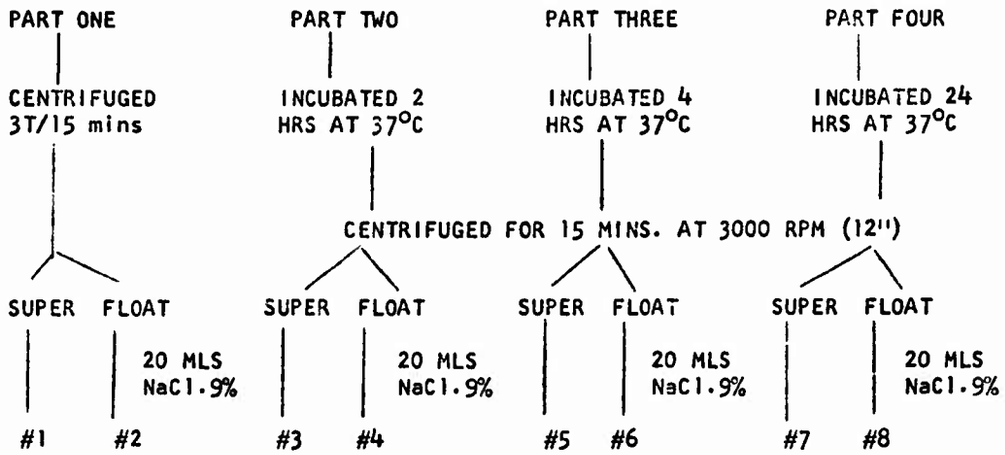
SAMPLE	DRY WT	TOXICITY IV	MGS LETHAL	# ANIMALS	TOXICITY IC	MGS LETHAL	# ANIMALS
N#1	16	LETHAL	1.6	2/2	LETHAL	0.48	5/5
N#2	14	LETHAL	3.5	2/2	LETHAL	0.21	6/6
N#3	16	LETHAL	1.6	2/2	LETHAL	0.48	3/5
N#4	30	LETHAL	0.74	2/2	LETHAL	0.45	3/5
N#5	10	LETHAL	0.24	2/2	LETHAL	0.30	2/2
N#6	2.5	NO REACTION		0/2	NO REACTION		0/5
N#7	UK	TOXIC		0/2	NO REACTION		0/3
N#8	14	******			LETHAL	0.42	5/8
N#9	30	******			LETHAL	0.90	3/5

NOTES: 0.2 Mls injected in IV and 0.03 Mls injected in IC. Dilution of toxic fractions with 10% Na-Heparin (1:1) did not affect toxicity.
****** = NOT DONE

EXPERIMENT 9 10-19-62; 11-1-62; 11-14-62

GENERAL METHOD

SKIN WAS DEFATTED, CHOPPED INTO FINE SECTIONS AND ADDED TO 2X VOL OF 96° C SALINE FOR 30 SEC. THEN HOMOGENIZED 1500 RPM/1 min. AND DIVIDED INTO FOUR EQUAL PARTS.



IN ALL OF THE ABOVE AFTER CENTRIFUGATION THE PRECIPITINS WERE DISCARDED.

SKINS TREATED AS ABOVE WERE SKIN Z COUNTY MORGUE NO POST NUMBER
SKIN AA COUNTY MORGUE 87 and 94 of AUG 62
SKIN DD COUNTY MORGUE 65 and 56 of NOV 62

IN ADDITION TO THE ABOVE GENERAL METHOD WITH SKIN AA, A PART OF EACH BEGINNING PART WAS INACTIVATED 1 HR AT 56° C. THIS RESULTED IN AN ADDITIONAL 8 FINAL SAMPLES WHICH WERE INACTIVATED COUNTER-PARTS OF THE ABOVE 8 SAMPLES. DATA ON THE TOXICITY OF ACTIVE AND INACTIVE SAMPLES ARE GIVEN IN TABLES ON THE FOLLOWING PAGES.

RESULTS EXPERIMENT 9

ACTIVE SAMPLES

SAMPLE	DRY WT MGS/ML	IV TOXICITY	MGS LETHAL	# ANIMALS	IC TOXICITY	MGS LETHAL	# ANIMALS
Z#1	15	LETHAL	1.5	1/1	NO REACTION		0/3
Z#2	76	LETHAL	0.4	5/6	NO REACTION		0/3
Z#3	17	LETHAL	1.7	1/3	NO REACTION		0/3
Z#4	89	LETHAL	0.6	5/5	SLIGHT REACTION	2.67	1/5
Z#5	18	LETHAL	1.8	1/2	NO REACTION		0/3
Z#6	83	LETHAL	2.0	1/1	TOXIC	2.49	0/5
Z#7	18	LETHAL	2.7	1/1	NO REACTION		0/3
Z#8	76	LETHAL	1.9	1/1	NO REACTION		0/3
AA#1	12	NO REACTION		0/2	**********		
AA#2	72	LETHAL	7.2	2/2	**********		
AA#3	12	LETHAL	1.2	2/2	**********		
AA#4	60	LETHAL	6.0	2/2	**********		
AA#5	14	LETHAL	2.8	2/2	**********		
AA#6	38	LETHAL	1.9	2/2	**********		
AA#7	16	LETHAL	3.2	2/2	**********		
AA#8	56	LETHAL	1.4	2/2	**********		
DD#1	22	LETHAL	0.5	2/2	NO REACTION		0/2
DD#2	101	LETHAL	1.7	2/2	SLIGHT REACTION	3.0	0/2
DD#3	17	LETHAL	1.7	2/2	NO REACTION		0/2
DD#4	108	LETHAL	1.4	2/2	LETHAL	3.0	3/5
DD#5	18	LETHAL	1.8	2/2	SLIGHT REACTION	0.54	0/3
DD#6	56	LETHAL	1.7	2/2	SLIGHT REACTION	1.68	1/5
DD#7	24	LETHAL	2.4	2/2	NO REACTION		0/2
DD#8	55	LETHAL	2.3	2/2	LETHAL	1.65	3/5

NOTES: 0.03 MLS INJECTED IC AND 0.2 MLS INJECTED IV. (IN SOME CASES) 0.1 IN OTHERS
**** = NOT DONE
IN ALL CASES MGS LETHAL REPRESENT MINIMUM MG AMOUNTS AT END OF DILUTIONS WHERE AS ALL PREVIOUS MG CONCENTRATIONS KILLED. WHERE NOT LISTED CONCENTRATED SOLUTIONS WERE INJECTED.

RESULTS EXPERIMENT 9

INACTIVE SAMPLES

SAMPLE	DRY WT MGS/ML	IV TOXICITY	MGS LETHAL	# ANIMALS	IC TOXICITY	MGS LETHAL	# ANIMALS
AA#1	16	LETHAL	3.2	2/2	**********		
AA#2	50	LETHAL	5.0	2/2	**********		
AA#3	14	TOXIC	2.8	2/2	**********		
AA#4	68	LETHAL	3.4	2/2	**********		
AA#5	**********						
AA#6	38	LETHAL	7.6	2/2	**********		
AA#7	16	TOXIC	3.2	2/2	**********		
AA#8	32	LETHAL	3.1	2/2	**********		

NOTES: SEE NOTES UNDER ACTIVE SAMPLE DATA

CRUDE FRACTIONS

EFFECTS OF INCUBATION AT 37°C OF THE SKIN-SALINE MIXTURES ON THE LETHALITY OF CRUDE SAMPLES

IV INJECTION OF THE SUPER

TIME IN HOURS	0	2	4	24
AVERAGE MGS LETHAL	3.06	1.5	2.5	2.7

IV INJECTION OF THE FLOAT

TIME IN HOURS	0	2	4	24
AVERAGE MGS LETHAL	1.1	2.6	1.8	1.9

IV = Intravenous injection in 18-22 gram mice - all in 0.2 ml volumes

SEE NEXT PAGE

CRUDE FRACTIONS

IV LETHALITY

COMPARISON OF LETHALITY IN FATTY FLOAT TO LETHALITY IN THE SUPER

HOT FATTY FLOAT			HOT SUPER		
# SAMPLES	% LETHAL	AVERAGE MGS LETHAL	# SAMPLES	% LETHAL	AVERAGE MGS LETHAL
23	95.7	2.47	23	82.7	1.97

MGS LETHAL RANGE IN FF 7.2 to 0.4

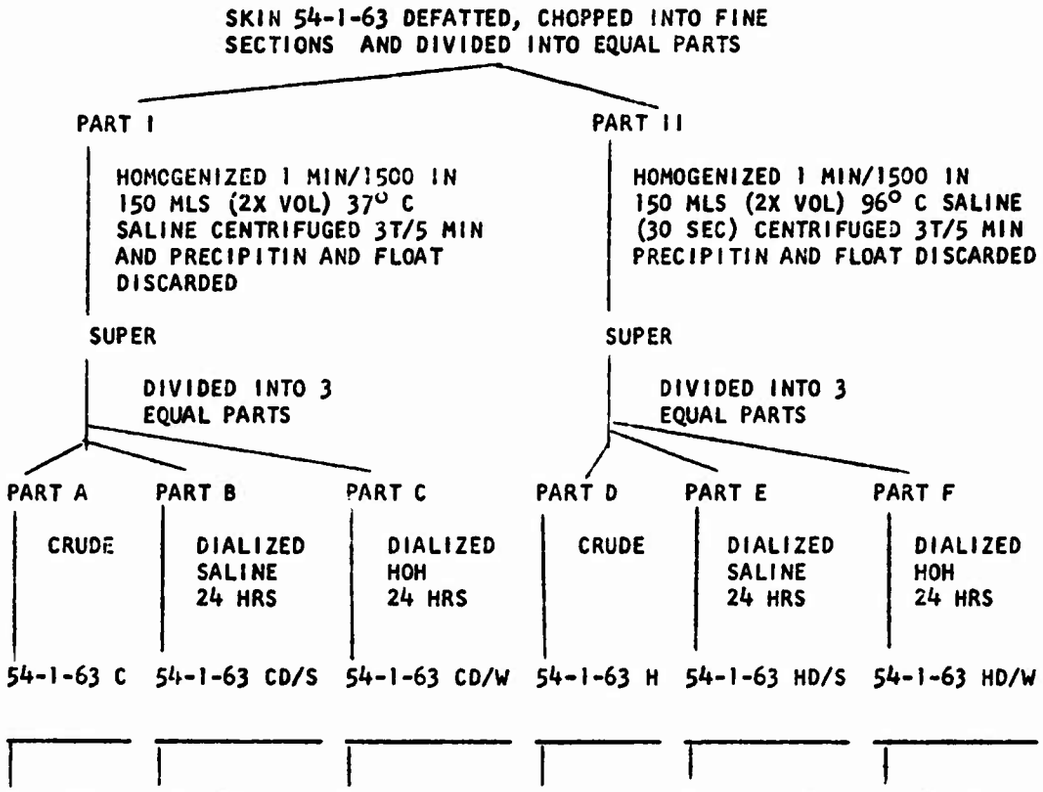
MGS LETHAL RANGE IN SUPER = 3.2 to 0.5

NOTE: ONLY MINIMUM MG LETHAL CALCULATED IN RESULTS.

IV = Intravenous injection in 18-22 gram mice, all in 0.2 ml volumes

EXPERIMENT 17 1-12-63

"CRUDE FRACTIONS: HOT VS COLD EXTRACTS"



ALL FRACTIONS WERE ADJUSTED TO 23 MLS IN VOLUME AND PRECIPITIN IN 80% ETOH AT RT. PRECIPITIN WAS REMOVED BY CENTRIFUGATION. PRECIPITINS WERE DIALIZED AGAINST HOH FOR 24 HRS (NUMBERS ARE SAME AS ABOVE BUT WITH ADDED NOTATION A/PPT). THE ETOH SUPERS WERE PERVAPORATED TO 5 MLS. (NUMBERS ARE SAME AS ABOVE BUT WITH ADDED NOTATION A/SUPER). IN BOTH CASES THE PRECIPITINS AND SUPERS WERE DIALIZED 24 HRS AGAINST HOH BEFORE TESTING TOXICITY. TOXICITY WAS TESTED ON NON DIFFUSIBLE MATERIAL ONLY.

TOXICITY RESULTS IN MICE ON FOLLOWING PAGE

RESULTS

"TOXICITY IN MICE"

SAMPLE	MG/ML DRY	I.V. TOXICITY 0.2 MLS I.V. 18-20 G MICE		
		VISUAL	MIN.MG LETHAL OR INJECTED	# DEAD / # INJECTED
54-1-63 C	13	Conc = No Reaction	2.6	0/5
54-1-63 CD/S	10	Conc = No Reaction	2.0	0/5
54-1-63 CD/W	3	Conc = No Reaction	0.6	0/5
54-1-63 H	21	Conc = Lethal 1:2 = Lethal 1:4 = No Reaction	4.2 2.1 1.5	2/2 3/3 0/2
54-1-63 HD/S	19	Conc = Lethal 1:2 = Lethal 1:4 = No Reaction	3.8 1.9 0.95	1/1 4/4 0/3
54-1-63 HD/W	11	Conc = Lethal 1:2 = Lethal 1:4 = Lethal 1:8 = No Reaction	2.2 1.1 0.55 0.27	1/1 1/1 4/5 /
54-1-63 C A/PPT	7	Conc = No Reaction	1.4	0/5
54-1-63 CD/S A/PPT	5	Conc = No Reaction	1.0	0/5
54-1-63 CD/W A/PPT	1	Conc = No Reaction	0.2	0/5
54-1-63 H A/PPT	13	Conc = Lethal 1:2 = No Reaction	2.6 1.3	4/4 0/1
54-1-63 HD/S A/PPT	12	Conc = Lethal 1:2 = No Reaction	2.4 1.2	4/4 0/1
54-1-63 HD/W A/PPT	10	Conc = Lethal 1:2 = No Reaction	2.0 1.0	4/4 0/1

NONE OF THE 54-1-63 C A/SUPER OR 54-1-63 H A/SUPER WERE TOXIC. TWO ANIMALS TESTED AT EACH STAGE.

COMPARISON OF HOT TO COLD EXTRACTS IN VARIOUS EXTRACTION METHODS

FRACTION	AVERAGE MGS HOT LETHALITY BY 18-20 GRAM MICE	AVERAGE MGS COLD LETHALITY BY 18-20 GRAM MICE
15 T PARTICLES	1.32	1.67
CRUDE	2.9	2.8
DIALIZED/SALINE	1.3	2.6
DIALIZED/WATER	0.6	0.9
CRUDE	1.3	1.4
DIALIZED/SALINE	1.2	1.0
DIALIZED/WATER	XXX	XXX

EXPERIMENT 24

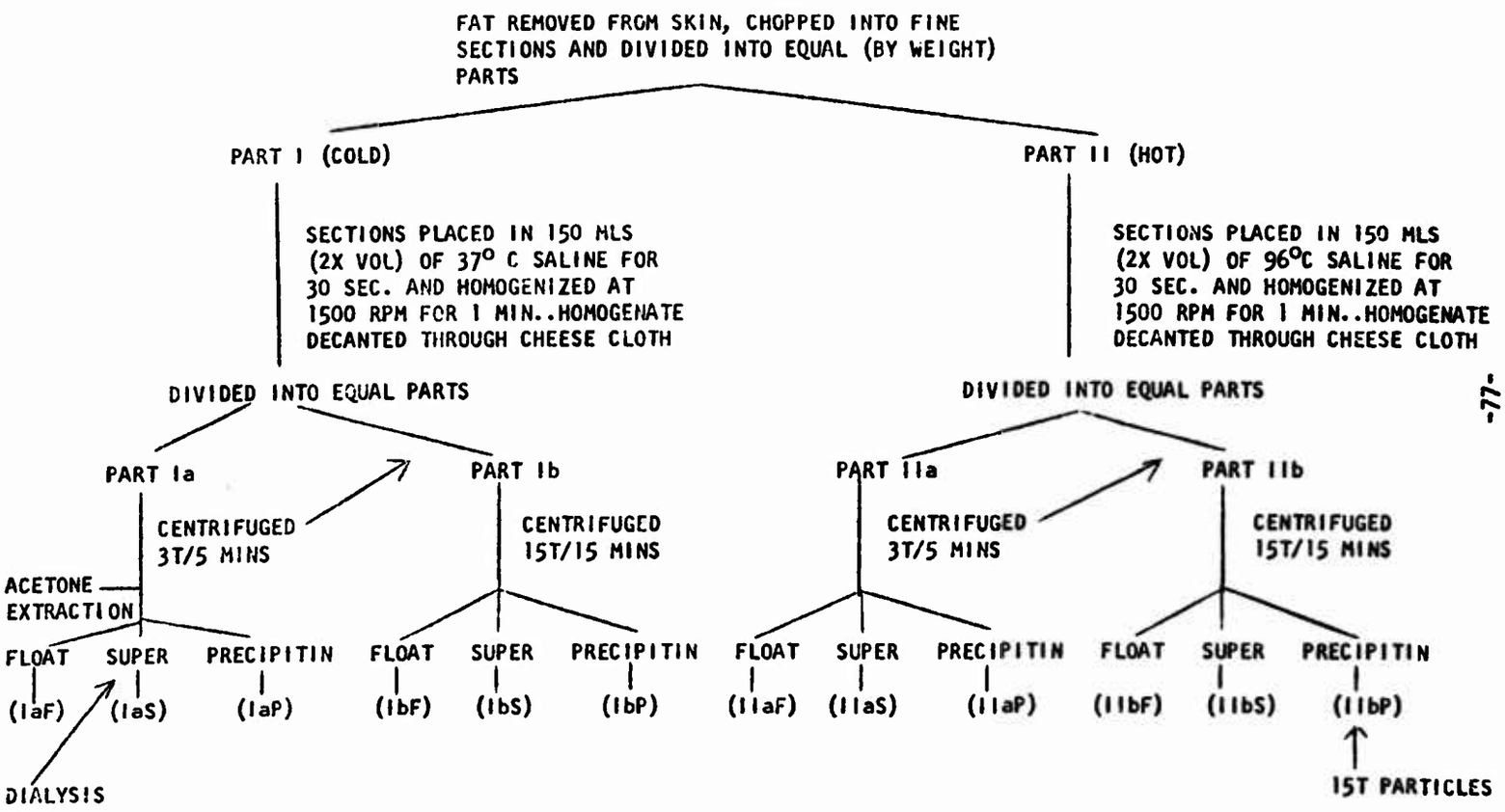
I. V. SALINE CONTROL EXPERIMENT

% SALINE	TOXICITY I.V. 0.2 MLS 18-20		
	VISUAL	MGS INJECTED	ANIMALS
1	NOT LETHAL	2.0	0/1
2	NOT LETHAL	4.0	0/1
3	NOT LETHAL	6.0	0/1
4	NOT LETHAL	8.0	0/1
5	SLIGHT	10.0	0/1
6	LETHAL	12.0	2/6
7	LETHAL	14.0	2/6
8	LETHAL	16.0	5/9
9	LETHAL	18.0	6/7
10	LETHAL	20.0	1/1

I.V. = Intravenous injection in 18-22 gram mice, all in 0.2 ml volumes

EXPERIMENT 25-26-27

EXPERIMENTAL OUTLINE (FRACTIONATION METHOD) 2-24-63 - GENERAL FRACTIONATION OUTLINE



EXPERIMENT 25 2-24-63 (A)

FLOW PER OUTLINE: SKINS 144, 150 and 169 of 2-63 COUNTY MORGUE

SAMPLE	DRY WT MG/ML	ACTIVE FRACTION, I.V. TOXICITY 0.2 MLS 18-20 G.			HEAT INACTIVATED FRACTION 56°C/30MIN I.V. TOXICITY 0.2 MLS 18-20 G.		
		VISUAL	MGS INJECTED	ANIMALS	VISUAL	MGS INJECTED	ANIMALS
IaF (A)*	22.5	LETHAL	2.2	6/6	SLIGHT	2.2	2/6
IaS (A)	12.2	LETHAL	1.2	5/7	NOT LETHAL	1.2	0/6
IaP (A)	**********						
IbF (A)*	27.0	LETHAL	2.7	6/6	LETHAL	2.7	3/3
IbS (A)	12.2	NOT LETHAL	2.4	0/4	**********		
IbP (A)*	11.1	LETHAL	1.1	6/6	LETHAL	1.1	3/3
IIaF (A)*	40.4	LETHAL	4.0	6/6	LETHAL	4.0	4/4
IIaS (A)	15.7	LETHAL	1.5	7/7	SLIGHT	1.5	1/6
IIaP (A)	**********						
IIbF (A)*	41.0	LETHAL	4.1	6/6	LETHAL	4.1	3/3
IIbS (A)	12.6	NOT LETHAL	2.5	0/4	**********		
IIbP (A)	6.0	LETHAL	0.6	5/6	NOT LETHAL	0.6	0/3

* = SEE NEXT PAGE FOR END DILUTION OF THESE SAMPLES AND HEAT INACTIVATION OF DILUTIONS

DATA CONTINUED

THE FRACTIONS STARRED ON THE DATA SHEET WERE CARRIED TO LOWEST POSSIBLE DILUTION. THESE LOW DILUTIONS WERE TESTED FOR HEAT INACTIVATION EFFECT AT 56° C FOR 30 MINS.

SAMPLE	ACTIVE		INACTIVE	
	MGS LETHAL	ANIMALS	MGS LETHAL	ANIMALS
IbF (A)	0.78	3/3	0.78	0/4
IbP (A)	**********			
IIaF (A)	1.0	4/4	1.0	0/4
IIbF (A)	1.0	4/4	1.0	0/4
IaF (A)	1.12	1/3	1.12	0/4

EXPERIMENT 25

EFFECT OF AGING ON LETHALITY OF EXTRACTS

32 DAYS AT 5° C.

SAMPLE	DRY WT MG/ML	ACTIVE FRACTIONS FROM EXPERIMENT 2-24-63 (A) I.V. TOXICITY 0.2 MLS 18-20 G.		
		VISUAL	MG INJECTED	ANIMALS
IbP	11.1	LETHAL	1.1	4/4
IIbP	6.0	LETHAL	0.6	5/5
IIbF	41.0	LETHAL	4.1	3/3
IIaS	15.7	LETHAL	1.5	5/5
IIaF	40.4	LETHAL	4.0	5/5
IIaS	16.0	LETHAL	0.4	4/5

EXPERIMENT 25-26-27

HOT COMPARED TO COLD (BOTH EXTRACTS FROM SAME SKIN SAMPLE)
ANALYSIS OF EFFECT OF VARIOUS TREATMENT ON CRUDE FRACTIONS

TREATMENT	3T SAMPLES						15T SAMPLES					
	HOT EXTRACTION			COLD EXTRACTION			HOT EXTRACTION			COLD EXTRACTION		
	FLOAT HOT 3T	SUPER HOT 3T	PPT HOT 3T	FLOAT COLD 3T	SUPER COLD 3T	PPT COLD 3T	FLOAT HOT 15T	SUPER HOT 15T	PPT HOT 15T	FLOAT COLD 15T	SUPER COLD 15T	PPT COLD 15T
CRUDE FRACTION (3)	LETHAL 0.179 17-88%	LETHAL 0.084 28-93%	X	LETHAL 0.210 16-38%	LETHAL 0.110 15-60%	X	LETHAL 0.233 13-100%	NOT LETHAL 0.147 14-0%	LETHAL 0.116 10-90%	LETHAL 0.266 13-61%	NOT LETHAL 0.142 14-0%	LETHAL 0.058 6-100%
HEAT INACTIVATED (3)	LETHAL 0.179 12-75%	LETHAL 0.084 28-75%	X	NOT LETHAL 0.116 6-30%	NOT LETHAL 0.110 13-15%	X	LETHAL 0.233 11-72%	X	LETHAL 0.079 8-62%	LETHAL 0.195 8-100%	X	LETHAL 0.058 3-100%
1 PART TO 1 PART WITH 10% HEPARIN(2)	LETHAL 0.164 4-100%	LETHAL 0.142 22-91%	X	X	LETHAL 0.163 3-100%	X	LETHAL 0.200 4-100%	X	LETHAL 0.200 4-100%	LETHAL 0.110 5-80%	X	X
ETHER EXTRACTED 3X AT RT AND 1X AT 42°C (1)	LETHAL 0.053 6-100%	LETHAL 0.100 4-100%	X	X	LETHAL 0.147 5-40%	X	LETHAL 0.063 3-66%	NOT LETHAL 0.147 3-0%	X	NOT LETHAL 0.147 5-0%	X	X
AGED AT 5°C 32 DAYS	LETHAL 0.210 5-100%	LETHAL 0.050 10-90%	X	X	X	X	LETHAL 0.216 3-100%	X	LETHAL 0.032 5-100%	X	X	LETHAL 0.058 4-100%
AGED AT 37°C 7 DAYS IN 1:10,000 MERTHIOLATE	LETHAL 0.185 5-100%	LETHAL 0.120 5-100%	X	X	NOT LETHAL 0.081 4-0%	X	X	X	LETHAL 0.126 5-100%	X	X	X

RING PRECIPITIN TESTS

According to Time of Performance

Underlays diluted 1:2 with saline; Overlays diluted 1:4 with saline(5 to 6 hour readings)

9-14-62	210 (A)	217 (A)	209 (A)	202 (N)	198 (A)
253 (HC)	+	+	-	-	-
212 (HC)	+	+	-	-	+
197 (C)	+	-	-	-	-
213 (HC)	+	+	-	-	+
186 (N)	-	-	-	-	-
TOTAL 25	A/C 9/16	N/C 0/4	A/N 0/4	N/N 0/1	

9-24-62	210 (A)	210 (A)	217 (A)	198 (A)	201 (N)
220 (C)	-	+	-	-	-
197 (C)	-	-	+	+	-
206 (C)	+	+	-	-	-
213 (C)	+	-	-	-	-
202 (N)	-	-	-	-	-
TOTAL 25	A/C 6/15	N/C 0/4	A/N 0/4	N/N 0/1	

(N) = Normal Sera
(A) = Acute Sera
(C) = Convalescent Sera
(HC) = Hospital Convalescent Sera

RING PRECIPITIN TESTS (cont'd)

10-6-62	218 (A)	215 (A)	215 (A)	217 (A)	216 (A)	198 (A)	202 (N)
188 (HC)	-	-	-	-	-	-	-
249 (HC)	-	-	-	-	-	+	-
206 (HC)	-	-	+	+	-	-	-
220 (C)	+	+	+	+	-	-	-
197 (C)	+	+	+	+	-	-	-
216 (C)	+	+	+	+	-	-	-
1326 (N)	+	-	-	-	-	-	-
1334 (N)	-	-	-	-	-	-	-
TOTAL 57	A/C 15/36	N/C 0/6	A/N 1/12	N/N 0/1			

10-9-62	218 (A)	215 (A)	215 (A)	210 (A)	198 (A)	1327 (N)
249 (C)	+	-	-	-	-	-
220 (C)	+	+	+	-	+	-
197 (C)	+	+	+	-	+	-
216 (C)	+	+	+	-	+	-
206 (C)	-	-	-	-	-	-
1340 (N)	+	-	-	-	-	-
1329 (N)	+	-	-	-	-	-
220 (C)	+	+	+	-	+	-
TOTAL 47	A/C 17/30	N/C 0/6	A/N 2/12	N/N 0/2		

RING PRECIPITIN TESTS (cont'd)

10-30-62

	239 (N)	218 (A)	215 (A)	215 (A)	217 (A)	209 (A)	233 (HC)	206 (HC)	231 (HC)
220 (C)	-	+	+	+	+	+	-	-	-
216 (C)	-	+	+	+	-	-	-	-	-
239 (N)	-	-	-	-	-	-	-	-	-
233 (HC)	-	-	-	-	-	-	-	-	-
206 (HC)	-	+	+	+	+	+	-	-	-
231 (HC)	-	+	+	+	+	+	-	-	-

TOTAL 54 — A/C 18/40 — N/C 0/5 — A/N 0/8 — N/N 0/1

11-1-62

	234 (A)	215 (A)	217 (A)	218 (A)	239 (N)
236 (HC)	-	+	-	+	-
248 (HC)	-	+	+	+	+
235 (HC)	-	+	+	+	-
231 (HC)	+	+	+	+	-
220 (C)	+	+	+	+	-
197 (C)	-	-	-	-	-
239 (N)	-	-	-	-	-

TOTAL 35 — A/C 16/24 — N/C 1/16 — A/N 0/4 — N/N 0/1

RING PRECIPITIN TESTS (cont'd)

11-13-62

	243 (A)	245 (A)	244 (A)	242 (A)	234 (A)	240 (N)	217 (A)	215 (A)	218 (A)
248 (HC)	-	-	-	+	-	-	+	+	+
235 (HC)	+	+	-	+	-	-	+	+	+
220 (C)	-	-	-	-	+	-	+	+	+
197 (C)	-	-	-	-	-	-	-	-	-
216 (C)	-	-	-	-	-	-	-	-	-
239 (N)	-	-	-	-	-	-	-	-	-

TOTAL 54 A/C 14/41 N/C 0/6 A/N 0/10 N/N 0/1

11-21-62

	270 (A)	218 (A)	230 (N)
220 (C)	-	-	-
236 (HC)	-	+	-
235 (HC)	-	+	-
240 (N)	-	-	-

TOTAL 12 A/C 2/6 N/C 0/3 A/N 0/2 N/N 0/1

11-27-62

	218 (A)	215 (A)	250 (A) P.M.	250 (A) Pre.	254 (A)	258 (N)	Moh.
220 (C)	+	-	+	+	+	-	-
236 (C)	+	+	-	-	+	-	-
235 (C)	+	+	-	-	+	-	-
240 (N)	-	-	-	-	-	-	-

TOTAL 29 A/C 10/19 N/C 0/3 A/N 0/6 N/N 0/1

RING PRECIPITIN TESTS (cont'd)

11-30-62	215 (A)	215 (A) (2)	254 (A)	304 (A)	256 (A)
236 (HC)	+	+	+	-	-
235 (HC)	+	+	+	-	-
247 (HC)	+	+	+	-	-
256 (N)	-	-	-	-	-
TOTAL 20	A/C 9/12	N/C 0/3	A/N 0/4	N/N 0/1	

1-8-63	269 (A)	268 (A)	263 (A)	267 (A)	277 (N)
272 (C)	+	+	+	+	-
276 (A)	-	-	+	-	-
TOTAL 12	A/C 4/4	N/C 0/1	A/N 1/4	N/N 0/1	

1-18-63	Moh (C)	265 (A)	264 (A)	253 (N)
Moh (C)	-	-	-	-
272 (C)	+	+	+	-
220 (C)	+	-	+	-
197 (C)	+	-	+	-
253 (N)	-	-	-	-
TOTAL 20	A/C 7/12	A/N 0/3	N/C 0/4	N/N 0/1

3-5-63	299 (HC)	297 (A)	290 (A)	291 (A)	294 (A)	284 (N)
Moh (C)	-	-	-	-	-	-
272 (C)	-	-	-	-	-	-
295 (C)	-	-	-	-	-	-
285 (N)	-	-	-	-	-	-
298 (HC)						
TOTAL 30	A/C 0/20	A/N 0/5	N/C 0/4	N/N 0/1		

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